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Ultraviolet A split-dose therapy, a novel approach to improve the aminolevulinate-based photodynamic therapy of skin lesions

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**Ultraviolet A split-dose therapy, a novel approach to
improve the aminolevulinate-based photodynamic therapy
of skin lesions**

Submitted by

Tina Radka

For the degree of

Master of Philosophy

University of Bath

Department of Pharmacy and Pharmacology

September 2010

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To my family..

TABLE OF CONTENTS

Acknowledgements	VII
Abstract	VIII
Abbreviations	IX
<u>Chapter One- Introduction</u>	1
1.1 Human Skin	1
1.2 Ultraviolet (UV) Radiation	4
1.2.1 General Information	4
1.2.2 Biological effects of solar (UV) radiation	5
1.2.2.1 Biological effects of solar UVA radiation	8
1.3 Oxidative stress and Reactive Oxygen Species (ROS)	13
1.3.1 Reactive Oxygen Species (ROS)	14
1.3.1.1 Superoxide radical anion ($\text{O}_2^{\cdot-}$)	16
1.3.1.2 Hydrogen Peroxide (H_2O_2) and hydroxyl radical (OH^{\cdot})	18
1.3.1.3 Singlet Oxygen ($^1\text{O}_2$)	20
1.3.2 UVA and ROS	22
1.4 Skin defence against UVA	26
1.4.1 Antioxidant Defences	27
1.4.1.1 Non-enzymatic molecules	27
1.4.1.2 Enzymes and Molecules	30
1.5 Iron	33

1.5.1 Body Iron	33
1.5.2 Labile Iron Pool (LIP)	35
1.5.3 Cellular iron homeostasis	36
1.5.4 Cellular iron uptake	38
1.5.5 Cellular iron storage	40
1.5.6 Iron homeostasis and oxidative stress	42
1.6 Photodynamic Therapy (PDT)	47
1.6.1 Definition	47
1.6.2 Cell/tissue damage mechanisms of PDT	48
1.6.3 First generation of photosensitisers	58
1.6.4 Second generation of photosensitisers	62
1.6.4.1 Phthalocyanines	63
1.6.4.2 Chlorin and Bacteriochlorin derivatives	65
1.6.4.3 Other porphyrin- and chlorophyll-derived photosensitisers	69
1.6.5. Topical photosensitising mediators: 5-Aminolaevulinic acid and –esters	73
1.6.5.1. Haem biosynthesis pathway	74
1.6.5.2 Advantages of topical ALA-PDT	76
1.6.5.3 ALA and ALA-esters uptake and cytotoxicity	77
1.6.5.4 Light sources for topical ALA/MAL-PDT	78
1.6.5.5. Dermatologic applications of ALA/MAL-PDT	82
1.6.5.6. Approaches to improve the uptake of ALA and MAL from skin lesions for efficient PDT response	86
1.6.5.7. Factors influencing the efficiency of ALA-PDT	88
1.6.5.8. Side effects of ALA- and MAL-PDT	92
1.7 Aims and objectives of the study	94

<u>Chapter two- Materials and Methods</u>	95
2.1 Chemical	95
2.2 Cell culture	95
2.2.1 Cell Model	95
2.2.2 Preparation of media	96
2.2.3 Trypsinisation	96
2.2.4 Experimental set up	96
2.3 Treatments	97
2.3.1 UVA irradiation	97
2.3.1.1 UVA lamps	97
2.3.1.2 UVA doses	97
2.3.1.3 Irradiation procedure	99
2.3.2 ALA treatment	99
2.3.3 Hemin treatment	99
2.4 MTT Assay	100
2.4.1 Principle of the assay	100
2.4.2 MTT stock solution	102
2.4.3 Procedure	100
2.5 Flow Cytometry	102

2.5.1 Principle of the assay	102
2.5.2 Procedure	102
2.6 Statistical analysis	103
<u>Chapter Three- Results</u>	104
3.1. Background	104
3.2 The effect of ALA on HaCaT cell survival following irradiation with single doses of UVA	105
3.3 The effect of ALA on HaCaT cell survival following irradiation with a second dose of UVA	108
3.4 The effect of hemin and ALA on HaCaT cell survival following irradiation with single or double split-doses of UVA	116
<u>Chapter Four- Discussion</u>	121
<u>Chapter Five- References</u>	130

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ABSTRACT

Topical aminolevulinate-based photodynamic therapy (ALA-PDT) is an effective method for treating skin lesions such as multiple actinic keratoses (AK). The major side effect of ALA-PDT is the pain experienced during the treatment. Management of treatment-related pain still remains a considerable challenge in patients. Further optimization of the treatment protocol including light source, dose and duration therefore seems crucial to try and alleviate pain. Previous data from this laboratory have demonstrated that ALA-treated human skin fibroblasts become highly sensitive to Ultraviolet A (UVA, 320-400nm)-induced cytotoxicity. In the present study, we provide further evidence that ALA-PDT with UVA is also effective in photokilling of human skin keratinocyte HaCaT cells despite their known resistance to UVA. The UVA-mediated damage in ALA-treated skin cells may be due to rapid release of potentially harmful transit labile iron (LI) in cells that in conjunction with increased generation of singlet oxygen ($^1\text{O}_2$) and other reactive oxygen species (ROS) by endogenously accumulated Protoporphyrin IX (PPIX) further exacerbate the oxidative damage in skin cells leading to cell death. We therefore hypothesized that by using UVA as the light source and by fractionating the dose applied into two short pulses with 1h dark interval, the ALA-treated skin cells could be further sensitized to UVA-induced damage, since the first dose of UVA would trigger the formation of ROS and extensive release of LI which in turn would intensify the damage caused by the second challenge dose of UVA. By using a series of UVA split-dose combinations, we could demonstrate that applying short pulses of UVA radiation to ALA-treated skin cells is a fast and efficient way to promote cell death. Furthermore the results of this project highlighted the importance of the first UVA dose in determining the efficiency of cell killing following the second UVA dose. The critical role of iron in exacerbating the UVA-induced damage of ALA-treated cells was confirmed with experiments showing that iron loading of cells with hemin prior to ALA-treatment could further sensitise the keratinocytes to very low doses of UVA. The damaging effects of short pulses of low UVA doses following ALA-treatment can be exploited to provide a rapid mean to improve the effectiveness of ALA-PDT of skin lesions while reducing considerably the therapy time and the discomfort/pain associated with prolonged high intensity visible light treatments.

ABBREVIATION

ADL	Argon-dye laser
AK	Actinic Keratoses
AlPcS2	Chloroaluminium sulphonated phthalocyanine
ALA	5-Aminolaevulinic acid
ALA-D	Aminolevulinate dehydratase
ALA-S	Aminolevulinate synthase
Apaf-1	Apoptosis protease activator 1
BCC	Basal Cell Carcinoma
BD	Bowen's disease
BPD-MA	Benzoporphyrin derivative monoacid
CSFs	Colony Stimulating Factors
Cyt C	Cytochrome C
DFO	Desferrioxamine mesylate (Desferal)
DMEM	Dulbeco's Minimum Essential Medium
DMSO	Dimethyl sulfoxide
DMT-1	Dimetal transporter-1
EDTA	Ethylenediaminetetraacetic acid
FCS	Foetal Calf Serum
FITC	Fluorescein isothiocyanate
Ft	Ferritin
GABA	gamma-aminobutyric acid
GPx	Glutathione peroxidase
GSH	Glutathione (reduced)
GSSG	Glutathione disulphide (oxidised)
H	Hour/hours

H-Ft	Ferritin heavy chain
HO	Heme-oxygenase
H₂O₂	Hydrogen Peroxide
HP	Haematoporphyrin
HPD	Haematoporphyrin Derivative
HPPH	2-[1-Hexyloxyethyl]-2-devinyl pyropheophorbide-a
ILs	Interleukins
IPL	Intense Pulsed Light
IRP	Iron Regulatory Protein
IRPs	Iron Regulatory Protein-1 and -2
IRE	Iron Responsive Element
LED	Light Emitting diode
L-Ft	Ferritin light chain
LI	Labile Iron
LIP	Labile Iron Pool
MAL	methyl-Aminolaevulinate
MH22	Murine Hepatoma
Min	Minutes
mBCC	morpheiform Basal Cell Carcinoma
m-THPBC	meta-tetra-hydroxyphenyl bacteriochlorin
m-THPC	meta-tetrahydroxyphenyl chlorine
MT	Metallothionein
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
NADH	Nicotinamide adenine dinucleotide (reduced)
NF-kappaB	Nuclear Factor kappa B
NMSC	Non Melonoma Skin Cancer

nBCC	nodular Basal Cell Carcinoma
NPe6	mono-L-aspartyl chlorine e6
$^3\text{O}_2$	Triplet Oxygen
O_2	Oxygen molecule
$\cdot\text{O}_2^-$	Superoxide radical anion
$^1\text{O}_2$	Singlet oxygen
$\cdot\text{OH}$	Hydroxyl radical
8-OHdG	8-hydroxydeoxyguanosine
PBG	Porphobilinogen
PBGD	Porphobilinogen deaminase
PBS	Phosphate buffered saline
PDD	Photodynamic diagnosis
PDL	Pulsed dye laser
PDT	Photodynamic Therapy
PI	Propidium Iodide
PPIX	Protoporphyrin IX
P/S	Penicillin/Streptomycin
PS	Phosphatidylserine
PUVA	Psoralen UVA
ROS	Reactive Oxygen Species
sBCC	superficial Basal Cell Carcinoma
SCC	Squamous Cell Carcinoma
SFM	Serum Free Media
SnET₂	Tin etiopurpurin
SODs	Superoxide dismutases
Tf	Transferrin
TfR	Transferrin receptor

TNF	Tumor Necrosis Factor
TPPS4	Tetra-sodium meso-tetraphenylporphine-sulfonate
TR	Thioredoxin reductase
TRx	Thioredoxin
UV	Ultraviolet
VPL	Variable pulse light

CHAPTER ONE

INTRODUCTION

1.1. Human Skin

Skin is the largest organ of the body, which keeps the internal systems intact. It is not only a barrier to protect the body from chemical and physical (e.g. ultraviolet) agents and micro organisms, but is also involved in defence mechanisms and other important functions. The skin thickness varies depending on the site of the human body, but the average is 0.3-0.4 mm. The skin is composed of two layers; the upper epidermis and the inner dermis (see **Figures 1 and 2**) (Bruls *et al*, 1984).

The epidermis begins at the outer surface and works inwards. There are four clear layers that can be observed in the epidermis under a light microscope. They are defined as:

- 1) Cornified layer - *stratum corneum*.
- 2) Granular layer - *stratum granulosum* - the zone where epidermal nuclei disintegrate.
- 3) Germinative or prickle cell layer - the bulk of living epidermal keratinocytes.
- 4) Basal layer - *stratum basale* - the only keratinocytes in normal epidermis that undergo cell division (Bruls *et al*, 1984).

The epidermis is mainly composed of keratinocytes with some Langerhans cells and melanocytes. Keratinocytes produce the protein keratin (which provides strength and

flexibility to the epidermis) and a low level of wide range of cytokines so that keratinocytes can influence immunologic function. These cytokines include interleukins (ILs), chemokines, tumour necrosis factor (TNF), colony stimulating factors (CSFs) and growth factors. Keratinocytes in the basal layer divide (on average every 4 weeks) and the daughter cells undergo changes as they move upward to the skin surface. At the final external layer (*stratum corneum*) dead cells are constantly shed, while new cells are continuously produced in the basal layer (Bruls *et al*, 1984). Langerhans cells are antigen presenting cells and play a major role in the immune surveillance system of the skin. Through maturation they become melanosomes and are then transported to the outermost layer of the epidermis. They have the role of forming a UV-absorbing barrier, which in turn reduces the amount of radiation that could penetrate the skin (Bruls *et al*, 1984).

The middle layer of the skin is the dermis, which is a connective tissue matrix between the epidermis and the subcutaneous layer. The upper dermis consists of capillaries which nourish the epidermis, and the lower thicker layer i.e. the reticular dermis. The major cell type in dermis is fibroblast, which synthesize elastin, collagen and glycosaminoglycans (GAG). Elastin fibres provide elasticity while collagen fibres provide strength. GAG provides viscosity, hydration and allows the dermis's limited movement. Other cells embedded in the reticular layer include fat cells and dermal dendrocytes, mast cells, macrophages and lymphocytes as well as many blood and lymphatic vessels, nerves and nerve endings, oil glands and hair roots (Carlson, 1994) (see **Figure 1**).

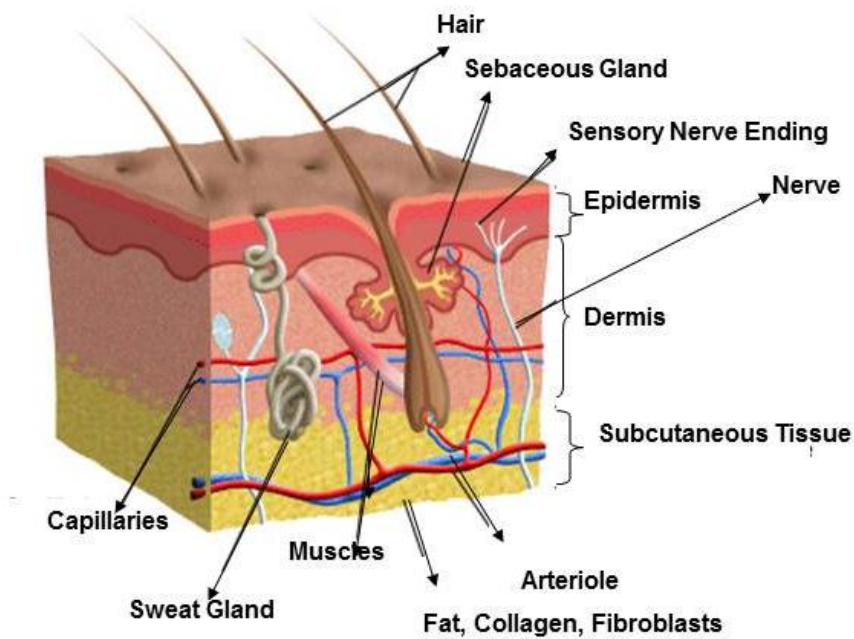


Figure1. Representation of skin layers and components.

(Modified from cancer.stanford.edu/.../tumorBiopsy.html)

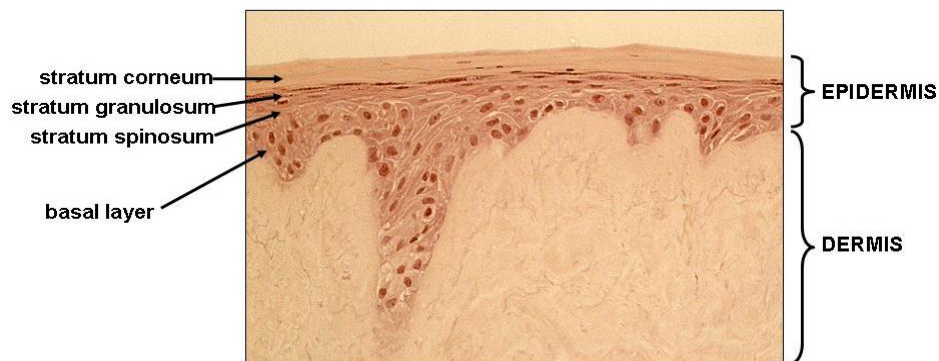


Figure 2. Representation of section of epidermal layers of skin as it can be observed under a light microscope. (Source: three dimensional organotypic skin culture made with normal human skin keratinocytes. (Aroun, Reelfs and Pourzand, unpublished data).)

1.2. Ultraviolet (UV) Radiation

1.2.1. General information

Ultraviolet (UV) is part of non-ionising electromagnetic radiation and includes wavelengths ranging from 100 nm to 400 nm (Tyrrell, 1994). Exposure to UV occurs from both natural and artificial sources.

The major source of natural radiation is sun. The sun emits radiation with wavelengths ranging from infrared (760-3000 nm) and visible (400-760 nm) to UV (190-400 nm). The UV component of sunlight is subdivided to UVA (320-400 nm, near-UV), UVB (290-320 nm, mid-UV) and UVC (190-290 nm) wavelengths (see **Figure 3**). Solar UV radiation comprises 5% of the total sun radiation emission. The majority of UV radiation of sunlight is UVA (>95%) (Tyrrell, 1994).



Figure 3. Presentation of solar electromagnetic radiation emission. The typical non- ionising electromagnetic source spectrum wavelength ranges from UV (UVC 190-290 nm, UVB 290-320 nm, UVA 320-400 nm) to visible (400-760 nm) and infrared (760-3000 nm) radiation. (Modified from <http://www.uvabcs.com/uvlight-typical.php>)

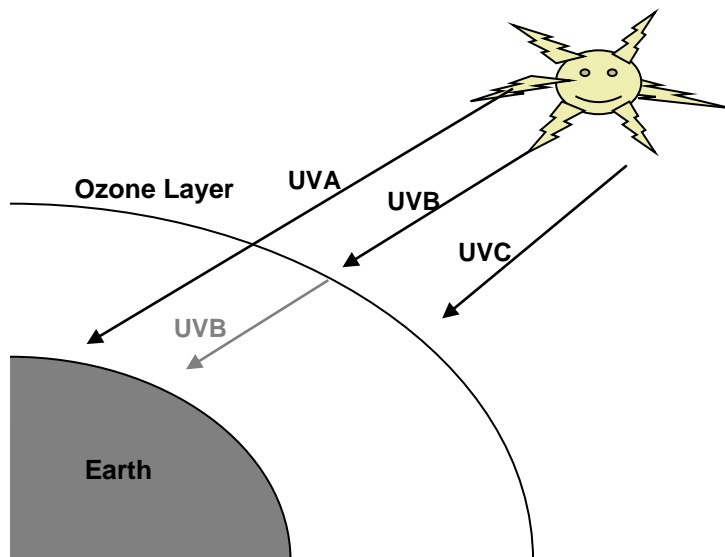


Figure 4. Representation of solar UV components and stratospheric ozone layer. UVC component of sunlight is filtered by ozone layer and cannot reach the surface of the earth. So the solar UV components reaching the earth's surface are composed of UVA and UVB only. (Modified from www.on.ec.gc.ca/.../tg_chap06_e.html)

A number of factors such as solar zenith angle, atmospheric ozone and cloudiness can influence the UV intensity reaching the surface of the earth. The spectral distribution of solar radiation that reaches the earth's surface is composed of visible, UVA and UVB components. The stratospheric ozone layer filters the UVC component of sunlight and as a result UVC does not reach the earth's surface (see **Figure 4**). Therefore, the effects of solar UV radiation on biological systems is limited to UVA and UVB wavelengths alone (Tyrrell, 2004).

1.2.2. Biological effects of solar UV radiation

The major targets for UV in humans are the skin and the eyes and the transmission of UV through these tissues and cells increases with increase in wavelength (see **Figures 5 and 6**).

Thus the longer wavelengths can penetrate deeper causing effects on targets which differ sharply from those of shorter wavelengths (Tyrrell, 1996).

The epidermis is the primary target for oxidative stress that is generated by both the solar radiation and also other chemical and physical agents in the environment. The amount of radiation received by the two major skin cells, the epidermal keratinocytes and dermal fibroblasts are different. The epidermal keratinocytes are exposed to both UVA and UVB radiation while the dermal fibroblasts are protected from UV radiation to a considerable extent by the overlying epidermis and therefore mostly receive UVA radiation (Tyrrell, 1994) (see **Figures 5 and 6**). A large proportion of UVA radiation can penetrate quite deeply into the skin and it has been shown that 35-50% of the overall UVA component of sunlight can reach the dermis of caucasian skin (Bruls *et al*, 1984). However only a small amount of UVA can reach below the surface of the skin and penetrate blood vessels (see **Figures 5 and 6**).

At cellular level, the interaction of UV with biological material changes as a function of wavelength and requires the absorption of the radiation by biomolecules. The UVB region overlaps with the DNA absorption spectrum and as a result the direct absorption of UVB by cellular DNA causes DNA photodamage and mutagenesis (Tyrrell, 1994; Freeman, 1989). In contrast, UVA is weakly absorbed by most biomolecules but is oxidative in nature, generating reactive oxygen species (ROS) such as singlet oxygen ($^1\text{O}_2$) via photochemical interactions with intracellular chromophores (Tyrrell, 1991; Tyrrell 1996).

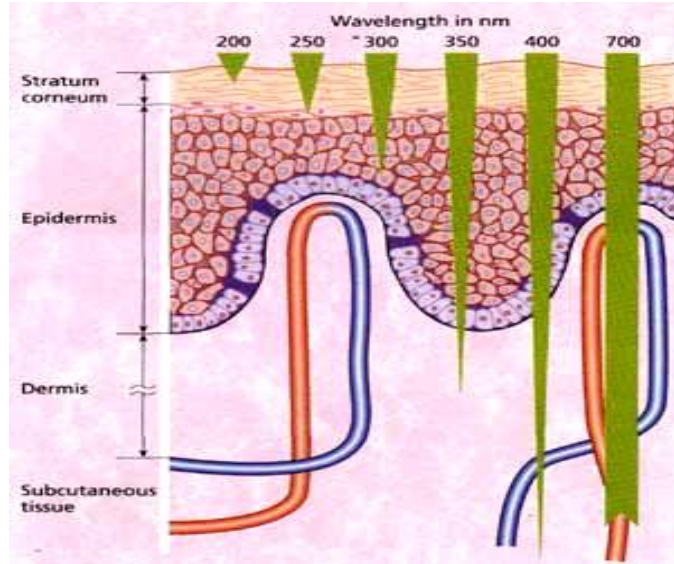


Figure 5. The depth of penetration of UV and visible wavelengths into the different layers of skin . The transmission of UV through skin layers increases with increase in wavelength. (Modified from www.pgbeautygroomingscience.com/the-sun.html).

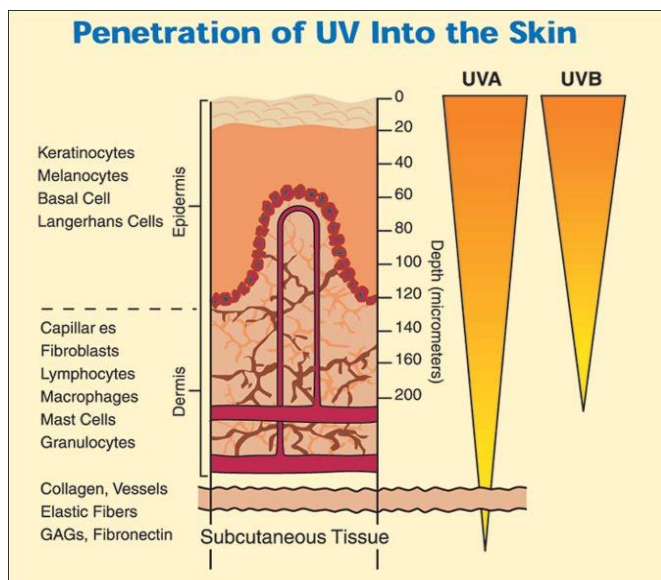


Figure 6. Penetration of solar UV radiation into the skin. UVA (320-400 nm) has a deeper penetration potential through the skin layers than UVB (290-320 nm). (Reproduced from Tyrrell, 1994 with permission)

Both acute and chronic exposures to sunlight are associated with various physiological and pathological states. The acute response involves immediate effects including erythema, heat, swelling, sunburn, pigmentation, hyperplasia, immune suppression and vitamin D synthesis (Gasparro *et al*, 1998). The chronic response involves delayed effects such as cataract and

skin ageing (also called photo-ageing), which is the result of morphological changes such as wrinkling, elasticity loss, uneven pigmentation due to general alteration of all the epidermal and dermal components of skin. Chronic exposures of skin to UV radiation may lead to skin cancer (reviewed by Tyrrell, 1994; Reelfs *et al*, 2010). In the recent decades, there has been a substantial decrease in the ozone layer and as a consequence the earth is exposed to more UV radiation. (Schwartz, 1997) This phenomenon has had an impact on human health in the form of increased incidence of sun-related skin disorders notably actinic keratoses (AK) and skin cancer (Schwartz, 1997) Nevertheless sunlight has several beneficial effects. The sun rays provide us heat, light and the general feeling of well being. The sunlight also stimulates blood circulation and the production of vitamin D that is required for maintaining blood calcium levels in individuals (Schwartz, 1997; Zanolli, 2004).

1.2.2.1. Biological effects of solar UVA radiation

The short-term effects of UVA on skin include erythema (skin redness), sunburn and pigmentation, and long-term effects include ageing and carcinogenesis (Tyrrell, 1991; Tyrrell, 1996). However UVA is about 10^3 - 10^4 fold less efficient than UVB to initiate these responses, as underlined by their action spectra (Parrish *et al*, 1982; De Gruijl and van der Leun, 1992). These ratios of efficiency also apply to other responses such as mutagenicity or lethality in cell cultures (Keyse *et al*, 1983; Tyrrell and Pidoux, 1987; Jones *et al*, 1987).

Most of the biological effects of UVA, either on cultured cells (Danpure and Tyrrell, 1976) or in skin (Auletta *et al*, 1986) are oxygen-dependent. Taking the action spectra data, solar spectro-radiometric measurements and known transmission of human skin into consideration have made us understand that the UVA component of sunlight contributes to up to 80% of the cytotoxic action of sunlight at the basal layer of epidermis (Tyrrell and Pidoux, 1987). Indeed

the greater histological effect of UVA is relatively observed on the dermis than on the epidermis for that for UVB. Human skin showed decreased permeability on the barrier of the *stratum corneum* when exposed to UVA (McAuliffe and Blank, 1991). UVA also depletes epidermal Langerhans cells, and recruits neutrophils into irradiated skin (Gilchrest *et al*, 1983).

At cellular level, UVA has been shown to cause lipid peroxidation at biologically relevant doses in the membrane of human cultured fibroblasts (Tyrrell, 1996). UVA-induced lipid peroxidation was found to be dependent on the “chemical” composition of membranes, as polyunsaturated fatty acid enrichment of human keratinocytes increases the peroxidation process (Quiec *et al*, 1995). Peroxidised membranes in cultured human skin keratinocyte tend to lose their fluidity following UVA irradiation. This suggested that loss of membrane integrity and selective permeability might result in alteration of transport systems, as well as the leakage of essential components or influx of extracellular molecules such as calcium and toxins.

This concept was backed up by the findings that UVA radiation inhibited in a dose-dependent manner both receptor-mediated and non-specific uptake of exogenous molecules (Djavaheri-Mergny, 1993). Internal lipid membranes of eukaryotic cells (e.g. lysosomal, mitochondrial and nuclear) have also been shown to be damaged following UVA irradiation. The latter has deleterious consequences to skin cells. UVA-induced damage to lysosomes is an early event that leads to temporary intracellular leakage of lysosomal proteases into the cytosol which in turn causes the degradation of cytosolic proteins notably the iron storage protein, ferritin (Ft) (Pourzand *et al*, 1999a). The UVA-induced proteolytic degradation of Ft leads to an immediate measurable increase in the level of potentially harmful redox active free transit iron pool, known as labile iron pool (LIP). The UVA-mediated increase in LIP has been shown to further exacerbate the peroxidative damage in cultured skin fibroblasts (Zhong *et al*,

2004) that may lead to the loss of cell membrane integrity. UVA also damages the mitochondrial membrane leading to immediate depletion of intracellular ATP. The depletion of cellular ATP along with loss of membrane integrity lead to necrotic cell death in irradiated skin cells (Zhong *et al*, 2004, see section **1.5.6**). Furthermore it was found that the slow kinetics of the induction of the nuclear transcription factor kappa B (NF-kappaB) by UVA relative to other oxidants is due to a transient increase in permeability of the nuclear membrane to proteins and occurs as a result of iron-mediated damage to the nuclear membrane (Reelfs *et al*, 2004). The apparent slow response of NF-kappaB to UVA radiation is likely to have consequences on the kinetics of activation of NF-kappaB target genes in the nucleus notably pro-inflammatory cytokines and proto-oncogenes (Tyrrell, 1996; Soriani *et al*, 1998; Reelfs *et al*, 2010).

Lipid peroxidation products may also induce damage to DNA (Vaca *et al*, 1988) as illustrated by the finding that lipid hydroperoxide decomposition products induced DNA adduct *in vivo* in liver and kidney (Wang and Liehr, 1995). Therefore, mutations may arise and alter gene expression.

In photodermatological studies, special attention has been given to “sunburn” keratinocytes, since the morphology of these cells is associated with characteristic features such as pyknotic nucleus and eosinophilic cytoplasm. The “sunburn” cells were first discovered in the epidermis of mammalian cells exposed to UVB radiation and later on regarded as an example of programmed cell death pathway now referred to as “apoptosis” (Weedon *et al*, 1979; Ley and Applegate, 1985; Young, 1987). This self-destructive programme became particularly important when it was shown that this process can eliminate pre-cancerous cells (Ziegler *et al*, 1994) and highlighted the necessity of understanding the phenomenon in order to develop therapeutic strategies for control of the carcinogenesis process (Barber *et al*, 1998).

Interest in the link between UV and apoptosis has increased since Godar *et al* (1994) investigated cell death mechanisms in different waveband regions of UV (UVC, UVB and UVA) on murine lymphoma cells. They found that all waveband regions of UV radiation cause apoptosis. However, UVA induced immediate (0-4h) and delayed apoptosis, whereas UVB or UVC induced delayed apoptosis (>20h). Studies from this laboratory have shown that unlike murine cultured cells, the human skin fibroblasts and keratinocytes are quite resistant to UVA-induced apoptosis and upon severe UVA insult, they die mainly by necrotic cell death (Pourzand *et al*, 1997; Pourzand and Tyrrell, 1999; Zhong *et al*, 2004; Reelfs *et al*, 2010).

DNA may be a target to UVA radiation, since it absorbs, although very weakly, in the UVA region up to 360 nm (Sutherland and Griffin, 1981). However, in contrast to UVB, UVA genotoxicity is most likely induced by indirect mechanisms involving absorption of photons by unidentified endogenous photosensitisers and generation of ROS (see section **1.3.2.**). This is suggested by (i) the oxygen-dependence of induction of most DNA lesions by UVA (Peak *et al*, 1987) and (ii) the fact that frequency of lesions induced by UVA does not follow the absorption spectrum of DNA, either *in vivo* (Freeman *et al*, 1989) or *in vitro* (Peak and Peak, 1995). Lesions include mainly DNA strand breaks and protein cross-links (covalent links between a protein and DNA). However, the formation of 8-hydroxydeoxyguanosine (8-OHdG), seems to be the most important type of lesion occurring in the UVA range and has been shown in different mammalian cell types (Kielbassa *et al*, 1997; Zhang *et al*, 1997). Most importantly the damage has been shown to depend on $^1\text{O}_2$ generation (Kvam and Tyrrell, 1997). This is the major interest since UVA radiation of sunlight produces biologically relevant levels of $^1\text{O}_2$ and also other ROS (Tyrrell, 1991) and the effects of UVA including cell inactivation are completely dependent on the presence of molecular oxygen

(Danpure and Tyrrell, 1976). Another type of damage, the pyrimidine dimers, has been shown to occur in human skin following UVA irradiation (Burren *et al*, 1998; Cadet *et al*, 2009).

Direct damage to proteins can happen at much longer wavelengths than direct damage to DNA. Although proteins absorb most strongly in the UVC range, as the wavelength is increased through the environmentally relevant UVB and UVA regions, damage to proteins become increasingly important relative to DNA damage because of absorption properties of the aromatic amino acids (tyrosine, tryptophan) which exhibit absorption that tails into UVA range (Vile and Tyrrell, 1995). Protein and amino acid hydroperoxides then produce various radicals via Fenton like reactions catalysed by iron metals, particularly ferrous iron (Neuzil *et al*, 1993; Dean *et al*, 1993).

Haem containing proteins (including cytochromes), the antioxidant enzymes catalase and peroxidases are potential targets for damage by UVA. Repair enzymes have also shown to be sensitive to UVA radiation and there is evidence that UV-induced repair disruption plays a role in cell death and mutagenesis (Haynes 1966; Webb *et al*, 1977; Menezes and Tyrrell, 1982). Oxidative modifications of for example collagen and transcription factors appear to mark them suitable for degradation in some systems (Helm and Gunn, 1986; Vince and Dean, 1987; Pacifici and Davies, 1990) but in others inefficient catabolism of oxidised proteins contributes to their accumulation (Wolff and Dean, 1986; Davies, 1986; Stadtman, 1992; Jessup *et al*, 1992; Grant *et al*, 1992).

Artificial sources of UV including UVA radiation have been used for the treatment of a number of diseases notably rickets, psoriasis, eczema and jaundice (Zanolli, 2004; Krutman, 2000). In view of the potentially harmful effects of UV radiation, the treatments take place only when benefits are thought to outweigh the risks.

This MPhil thesis investigates the potential of broad spectrum UVA radiation (320-400 nm) for photodynamic therapy of superficial skin lesions such as AK.

1. 3. Oxidative stress and reactive oxygen species (ROS)

Oxygen is by far the most abundant element in the earth's crust (Halliwell and Gutteridge, 1989). Oxygen is required for all the plants and animals for the efficient production of energy, except those organisms that are especially adapted to live under anaerobic conditions. Although oxygen is essential to life, it can give rise to a variety of ROS as part of normal metabolism (Halliwell and Gutteridge, 1989).

The term "oxidative stress" is referred to a situation in cells in which the equilibrium between pro-oxidant and antioxidant species is broken in favour of pro-oxidant state, due to generation of potentially harmful ROS (Halliwell and Gutteridge, 1989). Toxicities and pathologies associated with the oxidation of nucleic acids, proteins, lipids and carbohydrates have also been collectively termed "oxidative stress".

1.3.1. Reactive Oxygen Species (ROS)

A free radical is defined as “any atom or molecule with one or more unpaired electrons in an outer valence shell” (Halliwell and Gutteridge, 1989).

Free radicals are species capable of independent existence and contain one or more unpaired electrons in an orbital in the outermost electron shell. They are able to either take an electron from or donate an unpaired electron to another molecule. This property makes radicals very reactive towards their environment and therefore potentially damaging for living systems as they may be involved in the initiation and propagation of free radical chain reactions which potentially damage cells (Riley, 1994; Ryter and Tyrrell, 1998). Free radicals can kill bacteria, modify genomic and cellular structures, provoke immune responses, damage biomolecules, activate oncogenes, cause arterogenesis and enhance the ageing process (Halliwell and Gutteridge, 1989).

At the cellular level, the primary target of free radicals is the lipid bilayer of the membrane. The presence of polyunsaturated fatty acids in cell membranes as well as in intracellular membranes of organelles such as mitochondria and lysosomes makes them highly vulnerable to oxidative injury that results in lipid peroxidation. Lipid peroxidation is the process by which a free radical chain reaction will lead to generation of fatty acid radical and consequently fatty acid peroxy radical and aldehydes. The lipid peroxidation results in loss of cellular membrane integrity and leads to necrotic cell death (Girotti, 2001; Reelfs *et al*, 2010). Free radicals can also oxidize proteins and carbohydrates (Halliwell and Gutteridge, 1999).

Organic free radical species are numerous but oxygen-based free radicals which occur in biological systems are limited. ROS is a collective term that includes not only the oxygen-based radicals such as superoxide radical anion ($\cdot\text{O}_2^-$) and hydroxyl radical ($\cdot\text{OH}$) but also non-radical derivatives of oxygen such as singlet oxygen ($^1\text{O}_2$) and hydrogen peroxide (H_2O_2), which are able to form radicals. (Halliwell and Gutteridge, 1999).

ROS may be generated by exogenous or endogenous factors. Examples of exogenous factors include pesticides, ozone, photochemical smog, ionising and UV radiations. The endogenous mechanisms include mitochondrial respiration, microsomal and nuclear membrane electron transfer and phagocytic oxidative burst (Danpure and Tyrrell, 1976; Tyrrell and Pidoux, 1989).

ROS may be produced from the step by step reduction of O_2 molecule to H_2O as shown in

Figure 7.

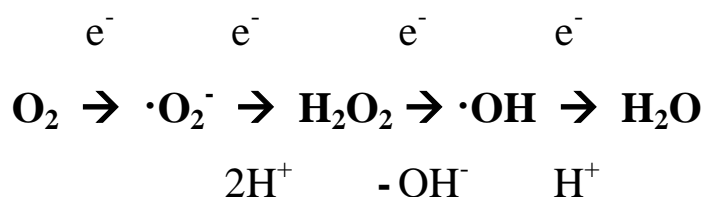


Figure 7: The series of four one-electron (e^-) uptake steps when O_2 is reduced to H_2O . The dots in two species $\cdot\text{O}_2^-$ and $\cdot\text{OH}$ indicate that they are radicals.

ROS, including $^1\text{O}_2$, may also be formed as a consequence of photochemical reactions between light, O_2 and light-reactive substances called ‘photosensitisers’ (Halliwell and

Gutteridge, 1990; Foote *et al*, 1982). The UVA component of sunlight produces $^1\text{O}_2$ and other ROS through the interaction of this penetration radiation with endogenous photosensitisers (i.e. chromophores) such as porphyrins in the skin (Tyrrell, 1994; Black, 1987; Trenam *et al*, 1992; Yasui and Sakurai, 2000).

The artificial photochemical generation of $^1\text{O}_2$ and other ROS by endogenous or exogenous photosensitisers are used to destroy malignant cells and tissues, in a process called photodynamic therapy (PDT). Biological $^1\text{O}_2$, however may also arise from non-photochemical sources such as the macrophage respiratory burst and membrane lipid peroxidation chain reactions (Cadenas, 1989; Krinsky, 1979).

Uncontrolled production of ROS causes damage to biomolecules and may in turn lead to diseases. ROS have been implicated in numerous types of cell and tissue injury, cell death (i.e. apoptosis or necrosis), ageing, drug action, drug toxicity as well as in initiation and progression of a number pathologic disorders notably chronic inflammatory disease and cancer (Halliwell and Gutteridge, 1999; De Groot, 1994; Stohs and Bagchi, 1995; Tenopoulo *et al*, 2005; Trenam *et al*, 1992).

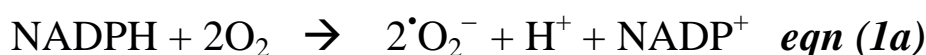
Some of the most important biologically relevant ROS are defined below:

1.3.1.1. Superoxide radical anion (O_2^-)

The superoxide radical anion (O_2^-) is the yield of one electron reduction of oxygen. The most important sources of O_2^- *in vivo* in aerobic cells are the electron transport chains of mitochondria during respiration and the endoplasmic reticulum. However, phagocytic cells (e.g. macrophages, neutrophils) generate directly O_2^- via an NADPH oxidase enzymatic systems (see *eqn 1a* or simplified *eqn 1b*) as part of their defence against invading microorganisms (Babior, 1999).

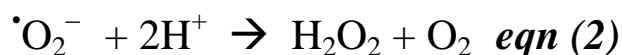
NADPH

Oxidase



The most important reaction of O_2^- with regard to living organisms is the dismutation reaction (*eqn 2*) which produces the well known oxidant H_2O_2 . This reaction is extremely slow in neutral solution (Crichton *et al*, 2008), but in cells it is extremely rapid due to catalysis by a group of enzymes, the superoxide dismutases (SODs).

SOD



Studies have shown that O_2^- may also be obtained through the UVA irradiation of NADH and NADPH *in vitro* (Czochralska *et al*, 1984; Cunningham *et al*, 1985).

$\cdot\text{O}_2^-$ is relatively unreactive towards most biomolecules, including lipids and nucleic acids (Fridovich, 1978). On the other hand, it may react with certain proteins and inactivate them, notably proteins containing transition-metal prosthetic groups such as iron-sulfur clusters (Gardner *et al*, 1997).

While several oxygen species act only as biological oxidants, $\cdot\text{O}_2^-$ may act as reductant as well (Koppenol, 2001). Thus $\cdot\text{O}_2^-$ is able to reduce iron from Fe(III) to its most reactive form Fe(II) (Biemond *et al*, 1988). This reducing capacity makes $\cdot\text{O}_2^-$ an important intermediate in the formation of $\cdot\text{OH}$ in the metal-catalysed Haber-Weiss reaction (see section 1.3.1.2.). Therefore $\cdot\text{O}_2^-$ toxicity to cells will depend largely on the availability of iron in the system.

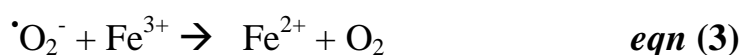
1.3.1.2. Hydrogen Peroxide (H_2O_2) and Hydroxyl Radical ($\cdot\text{OH}$)

The involvement of H_2O_2 in numerous types of cell and tissue injury is well-documented (Halliwell and Gutteridge, 1999; De Groot, 1994; Stohs and Bagchi, 1995; Tenopoulo *et al*, 2005). Although H_2O_2 itself has low reactivity towards cell constituents, it is capable of forming the highly reactive $\cdot\text{OH}$ in the presence of trace amounts of iron *via* the Fenton reaction.

Under physiological conditions, cells protect themselves either by the H_2O_2 –degrading enzymes catalase and glutathione peroxidase (GPx) (Pourzand *et al*, 2000), or by maintaining the level of redox active LIP via the cytosolic iron regulatory proteins 1 and 2 (IRPs) which function as post-transcriptional regulators of both iron uptake via transferrin receptor (TfR) and iron sequestration by the iron storage protein, Ft (Hentze and Kuhn, 1996; Cairo and Pietrangelo, 2000). However under pathological conditions, these defences are often

insufficient, because the system is either overwhelmed by an increased H₂O₂ formation (Rosser and Gores, 1995; De Groot, 1994; Sussman and Bulkley, 1990; de Groot and Brecht, 1991) and/or by an excess presence of labile iron (LI) (Tacchini *et al*, 1997). The simultaneous presence of excess redox active chelatable LI and H₂O₂ can be potentially toxic for cells as it can catalyze the formation of oxygen free radicals such as $\cdot\text{OH}$ via the Fenton reaction (Halliwell and Gutteridge, 1992).

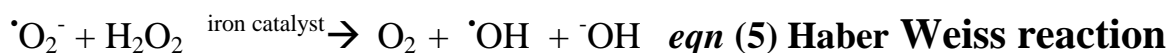
The major source of $\cdot\text{OH}$ generation is *via* the iron-catalysed Haber-Weiss or the Fenton reaction: In the first step $\cdot\text{O}_2^-$ reduces ferric iron to ferrous state:



Ferrous iron then reacts with H₂O₂ to generate $\cdot\text{OH}$:



Net reaction:



In cells the best catalyst of Fenton reaction is the low molecular weight LI, or alternatively the iron made available through other cellular sources under oxidative stress conditions such as UVA- and H₂O₂-induced release of LI from Ft (Jacobs, 1977; Puppo and Halliwell, 1988; Pourzand *et al*, 1999a; Breuer *et al*, 1997; Yiakouvaki *et al*, 2006).

The $\cdot\text{OH}$ radical is more reactive than $\cdot\text{O}_2^-$ and H₂O₂ but has considerably shorter half-life. In contrast, $\cdot\text{O}_2^-$ and H₂O₂ have longer half-lives but are less reactive (Martinez-Cayuela, 1995).

The highly reactive $\cdot\text{OH}$ radical is capable of interacting with most biomolecules including sugars, lipids, proteins and nucleic acids. These interactions that promote various harmful

processes in cells such as lipid peroxidation, protein oxidation, DNA/RNA oxidation and DNA lesions, ultimately overwhelm the cellular antioxidant defence mechanisms and lead to cell damage and cell death (Halliwell and Gutteridge, 1992 and 1999; Kakhlon and Cabantchik, 2002).

1.3.1.3. Singlet Oxygen ($^1\text{O}_2$)

In vitro studies have demonstrated that $^1\text{O}_2$ is a highly reactive form of molecular oxygen that may harm living systems by oxidizing critical organic molecules, including membrane lipid, protein, amino acids, nucleic acids, nucleotides, pyridine nucleotides, carbohydrates and thiols (Halliwell and Gutteridge., 1989). At the cellular level, the $^1\text{O}_2$ -mediated oxidation of macromolecules such as lipids could promote potentially harmful processes such as lipid peroxidation, membrane damage and cell death (Halliwell and Gutteridge, 1999).

$^1\text{O}_2$ is a derivative of molecular oxygen in which all valence electrons are spin-paired. It differs from ground state (triplet, $^3\text{O}_2$) molecular oxygen in the reversal of the spin direction of one electron in the outermost valence shell. This specific electronic configuration illustrates its non-radical nature (Kasha and Brabham, 1979).

$^1\text{O}_2$ exists in two forms of ground state and a higher energy form. The high form decays back to ground state configuration immediately upon formation and is thus thought to be irrelevant to biological systems (Ryter and Tyrrell, 1998).

The half life of $^1\text{O}_2$ is longer in deuterium oxide (D_2O) than in water or aqueous solution. Thus biological responses are often compared by replacing H_2O in the system with D_2O , as a test for $^1\text{O}_2$ generation (Pourzand *et al*, 2000).

As mentioned previously, $^1\text{O}_2$ (and other ROS) may be generated as a consequence of photochemical reactions between light, O_2 and light-reactive substances called

photosensitisers (Halliwell and Gutteridge, 1990; Foote, 1982). These include many natural substances such as porphyrins (hematoporphyrin and protoporphyrin IX), chlorophylls (*a,b*), bilirubin IXa, retinal (rhodopsin), quinones and flavins (riboflavin) (Spikes, 1989). The light might be absorbed by the photosensitiser in order to produce a chemical change. Indeed the first step in a photochemical reaction is the absorption of a single photon by the photosensitiser in the ground state (i.e. S^0) and the production of an excited state in which one electron in the outermost valence shell of the absorbing photosensitiser is raised to a higher energy level (i.e. S^{1*}) with conservation of spin direction. Because of the short lifetime of S^{1*} , it may either decay back to S^0 with emission of fluorescence or may be converted by intersystem crossing to the more stable excited triplet state (i.e. S^{3*}), where the promoted electron in a higher orbit undergoes a spin inversion. The generated S^{3*} is sufficiently long-lived to take part in chemical reactions and therefore the photochemical (or photodynamic) action takes place for the most part when in triplet state. There are two types of photochemical reactions. i.e. Type I and type II:

- Type I are electron- or hydrogen-transfer reactions between the triplet photosensitiser and other molecules resulting in production of potentially harmful ROS such as $\cdot O_2^-$, $\cdot OH$ and H_2O_2 and thereby returning of the photosensitiser to the ground state.
- Type II is an electron spin exchange between the photosensitiser in S^{3*} state and ground state triplet oxygen (3O_2) resulting in the production of 1O_2 while the photosensitiser returns to its ground state.

Both type I and type II reactions cause oxidation of biomolecules in the cell but 1O_2 is regarded as the main mediator of phototoxicity in PDT (Nyman and Hynninen, 2004; Calzavara-Pinton *et al*, 2007; Gomer *et al*, 1988; Gomer *et al*, 1989; Foote, 1991). Because the photosensitiser returns to the S^0 state in these reactions, it can generate a manifold concentration of reactive intermediates. Eventually, however, the photosensitiser is degraded

by light forming a photoproduct. This process, known as ‘photobleaching’ can results from reactions of type I or type II.

1.3.2. UVA and ROS

There is ample evidence that the biological effects of UVA radiation on cells are dependent on the presence of oxygen (Danpure and Tyrrell, 1976; Tyrrell and Pidoux, 1989), implying the involvement of ROS in UVA-mediated cytotoxicity. UVA is weakly absorbed by most biomolecules but is oxidative in nature, generating oxygen intermediates through a variety of photosensitisers, which are referred to as ‘chromophores’.

UV must be absorbed in order to produce a chemical change. Each chromophore is characterised by the wavelength at which it absorbs, a property which determines its relative importance at each wavelength throughout the UV spectrum. Absorption of UV radiation by a biomolecule leads to the excitement of its electron. The chemical modification of the biomolecule depends on its structure, the UV wavelength, and the conditions of the reaction occurred.

UV absorption by the biomolecule may create two outcomes:

1. Generation of reactive species in a metastable excited state.
2. Production of free radicals.

Both of the above are formed extremely quickly, since chemical reactions often occur within microseconds but may last for hours. These relatively fast processes are eventually translated into photobiological responses which could occur in seconds but can take years to become apparent (e.g. cancer).

In vitro, UVA irradiation of macromolecules has been shown to generate H_2O_2 , $\cdot\text{O}_2^-$ and hydroxyl radical $\cdot\text{OH}$ (Tyrrell, 1991). The highly reactive $\cdot\text{OH}$ can be generated via iron-catalyzed reduction of H_2O_2 by $\cdot\text{O}_2^-$ (Beauchamp and Fridovich, 1970).

In vivo, ROS may also be generated by UVA irradiation (Tyrrell, 1991; Beauchamp and Fridovich, 1970) via interaction with intracellular chromophores notably quinones, flavins, steroids and porphyrins, though the exact species remain to be defined (Tyrrell, 1994).

A number of reactions capable of generating $^1\text{O}_2$ under conditions relevant *in vivo* have been proposed (Badwey and Karvosky, 1980) and studies from Tyrrell's laboratory using either specific scavengers of $^1\text{O}_2$ or D_2O (which enhances the lifetime of this molecule) have clearly demonstrated the involvement of $^1\text{O}_2$ in UVA cytotoxicity to mammalian cells (Tyrrell, 1991; Pourzand *et al*, 2000). UVA effects also involve H_2O_2 formation and iron-catalysed generation of $\cdot\text{OH}$ (Tyrrell, 1991; Pourzand *et al*, 1999; Zhong *et al*, 2004; Reelfs *et al*, 2010). Based on such considerations, the UVA component of sunlight is now considered as a generator of intracellular oxidative stress.

Porphyrins and in particular protoporphyrin IX (PPIX), the immediate precursor of heme in heme biosynthesis is thought to be a very important UVA chromophore since it possesses a band absorption with a peak around 410 nm that tails with the UVA range and it has the potential of generating $^1\text{O}_2$ and other ROS upon UVA irradiation. This theory is supported by the fact that accumulating endogenous PPIX by exogenous aminolaevulinic acid (ALA) treatment strongly sensitises human skin fibroblasts (FEK4) and keratinocytes (HaCaT) to UVA photokilling (Pourzand *et al*, 1999b).

The light absorption properties of porphyrins have been used in a type of phototherapy called photodynamic therapy (PDT), based on addition of exogenous compounds (e.g. ALA) to

increase the level of endogenous photosensitisers (Kennedy *et al*, 1990; Rittenhouse-Diakun *et al*, 1995; Berg *et al*, 1996).

The pigment melanin which is confined to the epidermis and *stratum corneum* is also considered the main chromophore in human skin (Anderson and Parrish, 1981). Melanocytes are stimulated upon UVA irradiation and divide and synthesize melanin. Both forms of melanin, the reddish (phaeomelanin) and the black (eumelanin), take part in the screening effect of the whole epidermis. Reactive species can be generated from UVA irradiation of melanin, which may be harmful to the melanocytes and their cellular environment. For example human melanoma cells with high melanin content accumulated twice as much oxidative DNA damage upon UVA irradiation than cells with low melanin content (Kvam and Tyrrell, 1997). There is also evidence from *in vitro* studies suggesting that another important chromophore in the skin, the epidermal urocanic acid (a deamination product of histidine) may initiate chemical processes that could lead to the photoaging of the skin (Hanson and Simon, 1998). The amino acids tyrosine and tryptophan as well as NADH and NADPH, also exhibit absorption within the UVA range (Tyrrell, 1991).

$^1\text{O}_2$ and H_2O_2 are thought to be the most important ROS generated intracellularly by UVA, promoting biological damage in exposed tissues via iron-catalysed oxidative reactions (Vile and Tyrrell, 1995). It has been shown that physiologically relevant doses of UVA induce lipid peroxidation in membranes of human primary fibroblasts and keratinocytes via pathways involving iron and $^1\text{O}_2$ (Vile and Tyrrell, 1995; Morliere *et al*, 1991; Punnonen *et al*, 1991). Indeed, iron 'at' or 'near' strategic targets such as cell membranes, can undergo redox cycling by reacting sequentially with one electron reductants and oxidants, thereby generating toxic

oxidants such as $\cdot\text{OH}$ and lipid derived alkoxyl and peroxy radicals and can elicit biological damage (Aust *et al*, 1985; Halliwell and Gutteridge, 1992).

However in relation to UVA, current data from literature suggest that $\cdot\text{O}_2^-$ is not involved in any of the cellular effects mediated by UVA observed so far, including lipid peroxidation and protein oxidation (Vile and Tyrrell, 1995; Giordani *et al.*, 1997).

Immediate cellular effects of physiologically relevant doses of UVA include depletion of cellular glutathione (GSH) content, membrane lipid peroxidation and alteration in nuclear transcription factor activity and gene expression (Tyrrell, 1996; Vile and Tyrrell, 1995; Djavaheiri *et al*, 1996; Wlaschek *et al*, 1997; Klotz *et al*, 1997). The potentiation of UVA photokilling by GSH depletion provides further evidence for ROS involvement in UVA effects (Tyrrell and Pidoux, 1988). The UVA-induced generation of $^1\text{O}_2$ has been shown to play a crucial role in UVA-induced peroxidation of membrane lipids of cultured human skin fibroblasts as well as activation of nuclear transcription factors such as NF-kappaB (Gaboriau *et al*, 1995; Reelfs *et al*, 2004). Studies with iron chelators have demonstrated that iron-catalyzed ROS are also certainly involved in UVA-induced NF-kappaB activation, membrane damage and cell death (Reelfs *et al*, 2004; Zhong *et al*, 2004; Yiakouvaki *et al*, 2006; Reelfs *et al*, 2010).

The gene whose expression is most enhanced by UVA, encodes the mammalian stress protein and heme degrading enzyme, haem-oxygenase-1 (HO-1) (Keyse, 1989). Because of the generality of the response, the transcriptional activation of HO-1 is now used as a marker of oxidative stress in mammalian cells (Keyse and Tyrrell, 1989, Tyrrell, 1994). Studies with

D₂O (that enhances the lifetime of ¹O₂) and sodium azide and L-histidine (two quenchers of ¹O₂) have shown that ¹O₂ may be the primary effector in the transcriptional activation of HO-1 by UVA in cultured skin fibroblasts (Basu-Modak and Tyrrell, 1993). Further evidence for involvement of ¹O₂ in UVA-induced activation of HO-1 is provided by studies showing that ALA-mediated increase in endogenous photosensitiser PPIX strongly sensitizes the primary human skin fibroblasts to photokilling and drastically lowers the UVA dose threshold for activation of the HO-1 gene (i.e. from 50-100 to 5-10 kJ/m²) (Pourzand *et al*, 1999b; Rytter and Tyrrell, 1998).

A major consequence of UVA irradiation of human skin cells is the immediate release of chelatable 'labile' iron in the cytosol that appears to exacerbate the oxidative damage exerted by ROS generated by UVA. The UVA-mediated increase in LI in human skin fibroblasts plays a key role in activation of NF-kappa B and UVA-induced necrotic cell death (Pourzand *et al*, 1999b; Zhong, 2004; Pourzand and Tyrrell, 1999; Reelfs *et al*, 2004; Reelfs *et al*, 2010).

1.4 Skin defence against UVA

Stratum corneum, the physical absorption barrier of the skin, protects the epidermal keratinocytes and underlying fibroblasts to some extent (Pourzand and Tyrrell, 1999). Melanin can act as an additional defence of the epidermis. It provides some degree of physical protection and attenuates UV radiation by scattering and dissipation of absorbed energy (Tyrrell, 1994, Kollias *et al*, 1991). Since most cellular components of skin are susceptible to potentially deleterious oxidation, the cellular antioxidant systems are crucial to

the prevention or removal of the damage caused by the oxidising component of UV radiation. DNA repair and inducible protection pathways also contribute to cellular defence (Tyrrell, 1994).

1.4.1 Antioxidant Defences

As mentioned above, skin is the first interface with the external environment. As such it is extremely exposed to oxidative stress which generates reactive radical species directly or indirectly derived from the presence of oxygen. Due to susceptibility of cellular components to potentially harmful oxidation, cell survival could only happen by the existence of a large range of antioxidants defences, which are composed of free radical scavengers, either enzymatic or non-enzymatic, which sometimes act in synergy.

In vivo measurements in mouse and human skin have demonstrated that both enzymatic (catalase, glutathione peroxidase, glutathione reductase, and heme-oxygenase-2) and non-enzymatic (GSH, urate, ascorbate) antioxidant capacities of the epidermis is higher than that of the dermis (Shindo *et al*, 1993; Shindo *et al*, 1994; Applegate *et al*, 1995; Applegate *et al*, 1996). However UV radiation (UVB and UVA) reduces both enzymatic and non-enzymatic antioxidant defences in cultured skin cells (Pourzand and Tyrrell, 1999; Lautier *et al*, 1992; Sies, 1997; and Tyrrell, 1994). Possible mechanisms involved in the defence of the skin against UVA-mediated oxidative stress in the skin are presented in this section.

1.4.1.1. Non-enzymatic molecules

Glutathione (L-gamma-glutamyl-L-cysteinylglycine, GSH) is an important antioxidant molecule that is present in most mammalian cells in high concentrations (i.e. 3-5 mM). One

of its most important functions is to protect cells against oxidative damage (Meister and Anderson, 1983).

In human skin cells in culture, there is a direct correlation between the levels of endogenous GSH and sensitivity for cell killing by UVA (Tyrrell and Pidoux, 1986; Tyrrell and Pidoux, 1988). GSH levels modulate the levels of pre-mutagenic damage arising as a result of normal metabolism in cultured human cells and following UVA radiation (Applegate *et al*, 1992). It has been shown that in murine skin, GSH in both dermis and epidermis are depleted by UVA treatment (Connor and Wheeler, 1987).

The protection mechanism of GSH is unknown. However, as the most important intracellular thiol, it may act directly by scavenging radicals by hydrogen donation, competing with protein thiols for oxidising species, or indirectly as a cofactor for a number of protective enzymes including glutathione peroxidases (GPxs involved in detoxification of H₂O₂ or organic peroxides) (Ursini *et al*, 1982; Tyrrell and Pidoux, 1988; Lautier *et al*, 1992; Jornot and Junod, 1993). Many of the radical or non-radical reactions in cells involving GSH may lead to thiol oxidation to the disulphide, i.e., the oxidation of GSH to form GSSG. Therefore the reaction of reduction of GSH (catalysed by GSSG reductase), as well as the provision of essential reducing equivalents (NADPH) to this enzyme, are important in antioxidant defense.

Vitamin E (DL- α -tocopherol) is thought to be a major lipophilic non-enzymatic, free radical scavenger. Alpha-tocopherol acts as a chain breaking antioxidant in membranes by reacting with lipid peroxy radicals formed during lipid peroxidation. A number of studies have demonstrated that α -tocopherol can inhibit the UVA-mediated lipid membrane damage (e.g. Morliere *et al*, 1991; Gaboriau *et al*, 1993; Vile *et al*, 1995; Coulomb *et al*, 1996; Clement-Lacroix *et al*, 1996). *In vitro*, α -tocopherol is also capable of reacting and quenching ¹O₂

(Grams *et al*, 1972; Foote *et al*, 1974), however the importance of this phenomenon in biological membranes remains to be established.

Although the main *in vivo* function of α -tocopherol is to prevent lipid peroxidation, there is still very weak evidence for the photoprotective effects of vitamin E in animal cells and tissues (Bissett *et al*, 1990; Record *et al*, 1991; Fryer *et al*, 1993). Some protective effects have been reported in rodent cells in culture against UVB-induced cytotoxicity (Sugiyama *et al*, 1992), but not against DNA damage. Topical application of α -tocopherol acetate on the skin of mice prevented UVB-induced erythema and sunburn (Trevithick *et al*, 1992). UVA-induced cytotoxicity could be inhibited only in the case of a photosensitivity disease *i.e.* solar dermatitis (sun burn) by a water soluble vitamin E analogue, Trolox C, (Kralli and Moss, 1987). Nevertheless, in Pourzand's laboratory, it has been demonstrated that pre-treatment of cultured human primary fibroblasts with α -tocopherol-acetate could partially protect the cells against UVA-induced lysosomal damage and necrotic cell death (Julia Li Zhong, PhD thesis, 2002). Finally, there is evidence that vitamin E, at least when applied topically to the skin, is able to protect partially against ozone-mediated lipid peroxidation (Thiele *et al*, 1997).

Alpha-tocopherol is closely coupled to both vitamin C (ascorbate) and thiol cycle for the generation and maintenance of sufficient levels of cellular reducing power. The hydrophilic antioxidant, vitamin C (ascorbate) has a reducing ability (Berger *et al*, 1997). Furthermore, vitamin C may have a role in preventing oxidative damage by acting synergistically with vitamin E. The GSH-dependent free radical reductase may also generate oxidised vitamin E. A dietary antioxidant mixture (vitamin E, vitamin C and GSH) clearly reduced the UVB-induced tumour multiplicity and increased the tumour latent period in mouse studies (Black *et al*, 1985), demonstrating the importance of the concept of interaction between different

antioxidants. Ascorbate is able to react with a variety of active oxygen species (Halliwell and Gutteridge, 1989). It is, for example, able to quench $^1\text{O}_2$ (Chou and Khan, 1983), which is potentially an important way of protection in biological systems where $^1\text{O}_2$ is produced in the aqueous phase. However, ascorbate may also act as a pro-oxidant, since it can efficiently reduce iron Fe(III) to Fe(II), making it available for Fenton-type reactions. For example, ascorbate stimulates iron-dependent peroxidation of membrane lipids in certain circumstances (Muakkassah-Kelly *et al*, 1982; Basu-Modak *et al*, 1996).

Carotenoid pigments, such as vitamin A (β -carotene), are lipid-soluble compounds which can protect cells against photosensitised reactions in a few different ways (Krinsky and Deneke, 1982), including quenching of triplet sensitisers, quenching (inactivation) of $^1\text{O}_2$. This property is particularly important in the skin, since $^1\text{O}_2$ is probably the primary species generated by the interaction of UV/visible radiation with the photosensitizer PPIX present close to the skin surface. Beta-carotene has been proven to inhibit UV-induced epidermal damage and tumor formation in mouse models (Epstein, 1977; Mathews-Roth and Krinsky, 1987).

The role of the antioxidant in protecting cells against UV-induced oxidative stress requires further clarification. Since the long wavelengths in sunlight can penetrate through tissue and into blood, these defences may be critical under certain circumstances.

1.4.1.2 Enzymes and Molecules

The enzymatic system of the skin acts by catalysing the decomposition of oxidants and free radicals into less reactive species. Mammalian detoxifying enzymes include catalase, glutathione peroxidases/reductases, thioredoxin reductase and SOD. The haemoprotein

catalase specifically destroys H_2O_2 and is found in peroxisomes. Glutathione peroxidases (GPxs) and associated enzymes form a family of selenium-dependent haemoproteins which not only detoxify H_2O_2 but also reduce harmful hydroperoxides, such as those resulting from lipid peroxidation (Ursini *et al*, 1995). Peroxidases are found in the cytoplasm, peroxisomes and in the mitochondria. Superoxide dismutases (SODs), *i.e.* Cu, Zn-SOD, Mn-SOD and EC-SOD convert $\cdot\text{O}_2^-$ to H_2O_2 and are found respectively in the cytoplasm, mitochondria and bound to the extracellular matrix.

In cultured human cells, GPx and SOD are not affected by UVA radiation; however catalase is very sensitive to UVA and could be inactivated as a result of the radiation insult (Moysan *et al*, 1993; Tyrrell and Pidoux, 1989).

Enhancing GPx activity of cultured human cells by supplementing them with selenium provided protection against UVA-mediated damage (Leccia *et al*, 1993). Conversely, selenium deprivation of cells sensitized them to UVA and H_2O_2 -mediated cytotoxicity and lipid peroxidation (Bertling *et al*, 1996).

The thioredoxin/thioredoxin reductase (Trx/TR) system may also have a role in the cellular defence of skin against oxidative stress including that induced by UV radiation. Thioredoxin is a small protein which, in its reduced form, has a general protein disulfide reductase activity via its two reactive thiol groups (Holmgren *et al*, 1985). Thioredoxin reduces free radicals in human keratinocytes *in vivo* (Schallreuter *et al*, 1986). Thioredoxin expression is induced by oxidative stress, including H_2O_2 and UV (Spector *et al*, 1988; Nakamura *et al*, 1994) in a variety of cell types in culture including keratinocytes. The result is an increased protective capacity of the cell. A prognostic value for Trx has been described in malignant melanoma (Schallreuter *et al*, 1991).

Since iron has an important role as a catalyst in oxidative reactions, iron transport- and storage- proteins may play an important part as constitutive and/or inducible antioxidant defense, by keeping "circulating iron" low and in a non-toxic form. The intracellular storage protein Ft appears to play a critical role in this respect (see section **1.5**).

Similarly, owing to its metal binding capacity, metallothionein (MT) could contribute to skin protection against phototoxicity injury. In fact, metallothionein (MT) has been shown to be induced *in vitro* by UVC and UVB radiation (Stein *et al*, 1989; Hansen *et al*, 1997). Rodent cells with elevated levels of metallothionein have been shown to have increased resistance to UVA radiation (Dudek *et al*, 1993). The induction of expression of this gene also seems to correlate with a resistance to killing by several mutagenic agents. However, basal MT level may also function to regulate intracellular redox status in mammalian cells, since rodent MT null cells showed enhanced sensitivity to oxidative stress (i.e. tert-butylhydroperoxide) as compared to normal cells (Lazo *et al*, 1995).

Finally the copper-containing protein caeruloplasmin (both plasma and cytoplasmic forms) may represent an additional and distinct type of antioxidant behaviour, by oxidizing Fe^{2+} to Fe^{3+} (ferroxidase activity), thus preventing the iron from entering into a reversible redox system (Omoto and Tavassoli, 1990). This may facilitate iron incorporation by iron-binding proteins (Boyer and Schori, 1983; Samokyszyn *et al*, 1989).

1.5. Iron

1.5.1 Body Iron

Iron is the second most abundant metal, after aluminium and the fourth most abundant element in the earth's crust (5%) (Halliwell and Gutteridge, 1989). Living organisms, from bacteria to mammals, have selected iron to help them through vital biological processes, in which it is involved as part of, or as cofactor of proteins and enzymes (Aisen and Listowsky, 1980). Iron is a critically important metal for a wide variety of cellular events due to its important role in the active sites of a wide range of proteins involved in energy metabolism, respiration and DNA synthesis (Le and Richardson, 2002).

The total amount of iron in an average human body is about 4-5 g (Trenam *et al*, 1992), the majority of which is incorporated into the haem complex and can be found in proteins such as haemoglobin, myoglobin and cytochromes. The other type of iron is in the form of non-haem iron which is found in iron-sulfur cluster proteins such as IRPs as well as in iron transport protein transferrin (Tf), and iron storage protein Ft and hemosiderin (Cairo, 2006).

Iron is absorbed from the mammalian gastrointestinal tract by two protein-mediated mechanisms, one absorbing iron (II) and the other iron as haem (Shayeghi *et al*, 2005). Primates have evolved not to be able to excrete iron and, therefore, body iron levels are totally controlled by the absorption process (Andrews, 1999). The control of body iron is very important, because iron could be toxic when accumulated in excess. In the presence of molecular oxygen, 'loosely-bound' iron is able to redox cycle between the two most stable oxidation states, iron (II) and iron (III), thereby generating oxygen-derived free radicals such as $\cdot\text{OH}$. The pathological consequence of iron-catalyzed oxidative damage are recognized in diseases such as hepatitis, haemochromatosis, liver cirrhosis, cancer and neurodegenerative

disease (Andrews, 1995; Gaeta and Hider, 2005; Kalinowski and Richardson, 2005; Molina-Holgado *et al*, 2007; Bacon and Britton, 1990; Kehrer, 2000; Kowdley, 2004; Valko *et al*, 2006; Valko *et al*, 2007). Excess iron may also aggravate diabetes, cancer, cardiovascular disease and alcoholic and non-alcoholic steatohepatitis (Swanson, 2003; Brewer, 2007; Kongho *et al*, 2005; Peterson, 2005; Imeryuz *et al*, 2007). The presence of excess iron has also been demonstrated in a variety of skin disorders such as psoriasis (Molin and Wester, 1973), venous ulceration (Ackerman *et al*, 1988) and atopic eczema (David *et al*, 1990), indicating the involvement of iron in the pathology of skin. Iron may also play a role in the carcinogenic process of other transition metals such as copper, or other types of carcinogens (Toyokuni *et al*, 1996).

About 60% of the total body iron is incorporated in haem of haemoglobin in the circulating red blood cells for oxygen transport and 15% in the muscles in the form of myoglobin (Crichton *et al*, 2008). Only a very small amount of the total body iron is present in functional iron, as an essential component of a very large number of heme (*e.g.* cytochromes, catalase, oxidases and peroxidases) and non-haem proteins and enzymes (*e.g.* ribonucleotide reductase, oxidases and iron sulfur-proteins). Most of these proteins are required for normal cellular metabolism and are involved in important processes such as oxidative production of cellular energy, electron transfer and DNA biosynthesis. All of these may account for no more than 10% of the total iron pool. The remaining 15% can be found principally in the cytoplasm in the form of the iron-storage protein Ft but also in lysosomes, as hemosiderin. Storage iron is found in the liver, mainly in the hepatocytes, but it is also found in macrophages in the liver, bone marrow, spleen and muscles, where it is readily available as a reserve in case of blood loss (Kuhn, 1994; Crichton *et al*, 2008).

All these iron-complexing molecules leave body fluids and cells with extremely low concentration of free transit iron. Nevertheless there is now strong evidence for the existence of a transit pool of catalytically active free iron known as labile iron pool.

Indeed in contrast to iron bound to proteins, the intracellular labile iron can be potentially toxic, especially in the presence of ROS, as it can lead to catalytic conversion of 'poorly reactive' oxygen species into 'highly reactive' and damaging oxygen-derived free radicals such as $\cdot\text{OH}$ radical and lipid-derived alkoxyl and peroxy radicals (Halliwell and Gutteridge, 1990; Halliwell and Gutteridge, 1992). The highly reactive $\cdot\text{OH}$ radical species are capable of interacting with most types of biological material including sugars, lipids, proteins and nucleic acids. These interactions that promote various injurious processes in cells such as lipid peroxidation, protein oxidation, DNA/RNA oxidation and DNA lesions, ultimately overwhelm the cellular antioxidant defense mechanisms and lead to cell damage and cell death.

1.5.2. Labile iron pool (LIP)

The cellular LIP is defined as a pool of redox-active iron complexes and it was first suggested by Jacobs (1977) as an intermediate or transitory pool between extracellular iron and cellular iron associated with proteins. Iron belonging to this intracellular pool comprises both ionic forms of iron (Fe^{2+} and Fe^{3+}) loosely bound to low molecular weight ligands such as organic anions (phosphates and carboxylates), polypeptides and surface components of membranes (e.g. phospholipid head groups) (Kakhlon and Cabantchik, 2002; Kruszewski, 2003). Labile iron pool is accessible to permeant chelators and is considered metabolically and catalytically reactive (Breuer *et al*, 1996; Epstein *et al*, 1997; Cairo and Pietrangelo, 2000; Petrat *et al*, 2001). This definition implies that LIP can not only potentially participate in redox cycling

but also be scavenged by permeant chelators. The latter property forms the basis for the quantification of the cellular LIP, which in quiescent conditions comprises only minor fractions of the total cellular iron (< 5%) (Kakhlon and Cabantchik, 2002). Originally, LI was considered to be cytosolic, however recent studies have indicated that only a minor fraction of the total cellular redox-active iron resides in the cytosol. The quantitative studies of LIP in various cell lines using a series of fluorescent iron probes have shown that lysosomal compartments have the highest proportion of LI followed by mitochondria that have much superior level of LI when compared to cytosolic pool (Petrat et al, 2000; Petrat *et al*, 2000, 2001; Fakhri *et al*, 2008).

1.5.3. Cellular iron homeostasis

The level of LIP within cells must be maintained so that there is adequate iron for the cell's requirement but not so much that it becomes toxic. Alterations in LIP are normally sensed by the cytosolic iron IRP1 and IRP2 which function as post-transcriptional regulators of both iron uptake via the TfR and iron sequestration by the iron storage protein, Ft (see **Figure 8** and **9**):

When iron is scarce in the LIP, Ft and TfR mRNAs are specifically recognized and bound by the active forms of IRPs (IRP1/2), leading to stabilization of the TfR mRNA and inhibition of Ft translation, both of which will lead to enhanced levels of LIP.

Conversely, during an increase in iron supply, IRPs are converted to low affinity mRNA-binding proteins, leading to the induction of Ft mRNA translation and the degradation of TfR mRNA, which will ultimately lead to a reduction in the LIP (Klausner *et al*, 1993; Beinert

and Kennedy, 1993; Kuhn, 1994; Guo *et al*, 1995; Pantopoulos, 2004; Hentze and Kuhn, 1996).

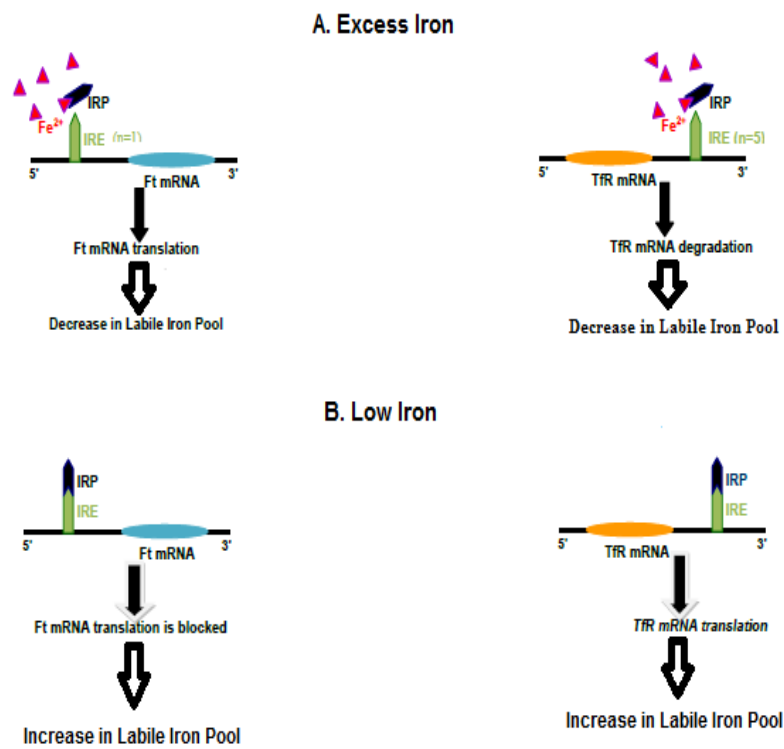


Figure 8. The reciprocal translational regulation of transferrin receptor (TfR) and ferritin (Ft) by IREs and IRPs. (Modified from Yiakouvaki, 2003 PhD thesis).

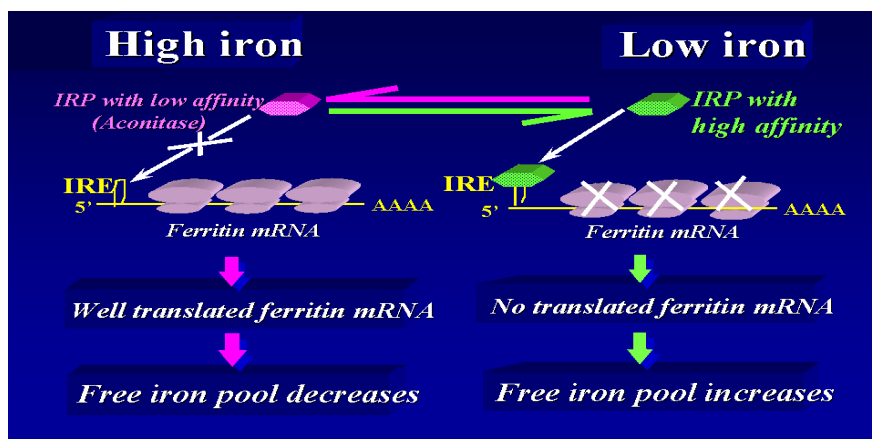


Figure 9. The schematic presentation of regulation of Ft mRNA translation by IRP-1 during high and low intracellular labile iron conditions. (Reproduced from C. Pourzand's presentation slide, with permission) (Pourzand *et al*, 1991a).

1.5.4. Cellular iron uptake

Within the body the supply of iron to tissues and cells is mediated by two principal proteins, namely transferrin and TfR.

Transferrin (Tf) is the plasma iron-binding glycoprotein which is considered as the main source of iron transfer in the body. It is responsible for iron supply for biological needs of iron requiring cells and is considered the only available source of iron for haemoglobin synthesis (Cairo *et al*, 2006). Its function is to reduce the toxicity of iron by binding and transporting iron. The 80kDa Tf has high affinity for Fe^{3+} , and it consists of two globular domains, each of which can independently bind a single ferric ion. In conditions of normal iron status, serum Tf is saturated to about one third of its iron-binding capacity, such that a mixture of apo-Tf (iron free), the two monomeric forms (one iron) and diferric-Tf (two iron) is present.

The 180kDa TfR is a glycoprotein which mediates the entry of diferric-Tf from the extracellular compartment into the cells (Eisenstein and Blemings, 1998). This glycoprotein is a disulfide-linked homodimer composed of two identical glycosylated subunits. The subunits are transmembrane polypeptides of 760 amino-acid residues with a short N-terminal cytoplasmic domain, a single transmembrane region and a large glycosylated portion, which contains the Tf-binding site. Up-regulation of the expression of cellular TfR occurs as a result of an inadequate tissue supply of iron or increased cellular demand for iron. As a result, elevation of soluble form of TfR can be found in the context of iron deficiency diseases. There are two forms of TfR, named TfR1 and TfR2 which are thought to have distinct cell and tissue specific expression pattern. (Kawabata *et al*, 1999; Levy *et al*, 1999). TfR is expressed at high levels in rapidly dividing cells (Kalinowski and Richardson, 2005).

Although, in normal subjects, Tf is made up of different proportions of the four Tf species described above, it is predominantly the diferric-Tf ($\text{Fe}_2\text{-Tf}$) molecule that is utilised by the cell.

Figure 10 summarises the mechanism by which cells uptake iron from Tf via TfR (Andrews, 1999). Briefly the $\text{Fe}_2\text{-Tf}$ molecule binds to the TfR at the plasma membrane. The transferrin-transferrin receptor ($\text{Fe}_2\text{-Tf-TfR}$) complex are localised in clathrin-coated pits, which pinch off from the membrane, and after losing their clathrin coats, they fuse with the target membranes of endosomes delivering the vesicle contents into the interior of the endosome. An ATP-dependent proton pump maintains the pH of the endosome at around 5-6. This intracellular compartment functions as a sorting station in the inward pathways of extracellular molecules into the cell, known as receptor-mediated endocytosis. The early endosomes containing $\text{Fe}_2\text{-Tf-TfR}$, are rapidly sorted, enabling them to escape from subsequent lysosomal degradation. Conformational changes of the $\text{Fe}_2\text{-Tf-TfR}$ complex at mildly acidic pH within the endosome lead to release of iron from the complex as Fe^{3+} . The

apoTf-TfR complex is returned to the cell surface, where the complex dissociates, allowing both components to be reutilised in further cycles of cellular uptake of Tf iron. The endosomal Fe^{3+} which has been released from the $\text{Fe}_2\text{-Tf-TfR}$ complex must be reduced to Fe^{2+} before the divalent metal ion transporter (DMT1) can transport it across the endosomal membrane into the cytoplasm. The identity of the ferrireductase involved in this process remains unknown. Once in the cytoplasm, the Fe^{2+} enters the intracellular transit iron pool, referred to as LIP. In erythroid cells most of this iron enters the mitochondria, where it is incorporated into heme, whereas in non-erythroid cells, iron from the LIP can be stored in Ft.

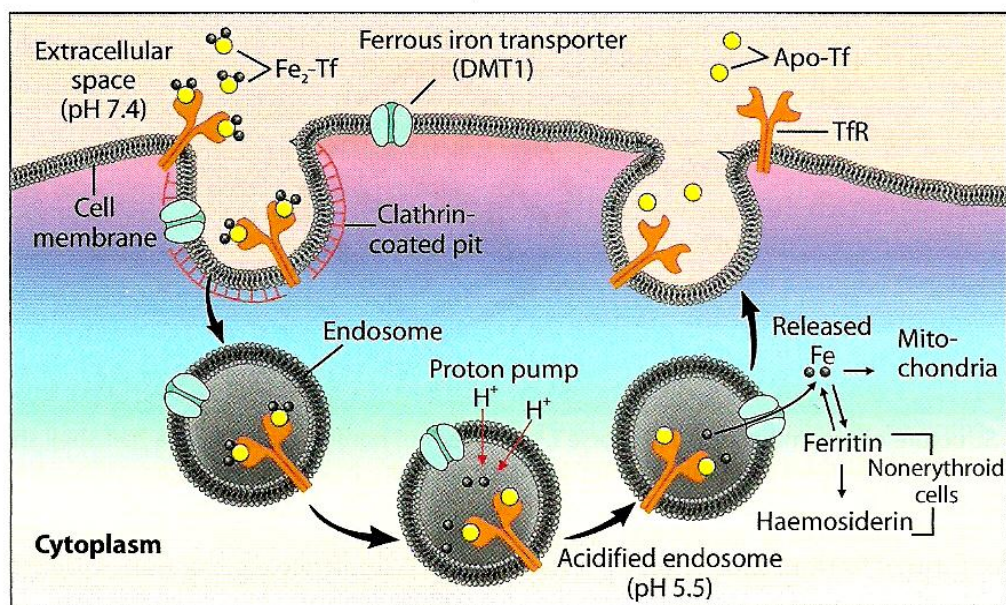


Figure 10. Cellular iron uptake. (modified after Andrews 1999). Iron bound to Tf is internalised into cells by TfR-mediated endocytosis. Iron is then released from Tf-TfR complex in endosomes and is transported across the endosomal membrane by DMT1 which is present in endosomal membrane. Once in cytosol iron enters the intracellular LIP and can be either stored in Ft (i.e. in non-erythroid cells) or enter mitochondria for heme synthesis (i.e. in erythroid cells).

1.5.5. Cellular iron storage

Within the cell, iron can be stored in two forms, in the cytosol as Ft and after breakdown of Ft within the lysosomes, as hemosiderin. Ft is an oligomeric protein which forms a hollow protein shell within which substantial amounts of iron can be stored in a non-toxic, soluble and bioavailable form. In contrast, hemosiderin is water-insoluble, contains a much higher ratio of iron to protein and can be directly visualised histologically by its characteristic Prussian blue reaction (i.e. the Perl's stain) (Crichton *et al*, 2008).

Most of the iron that is not metabolised is stored in the cytosolic iron storage protein, Ft. Ft is an ubiquitously expressed hollow protein which is made up of 24 polypeptide subunits. The heteropolymeric components (light chain, L, 19 kDa; and heavy chain, H, 21 kDa) form a shell around an inorganic iron oxyhydroxide core $[\text{FeOOH}]_x$. The sequestered iron is maintained in an oxidised Fe^{3+} state by the ferritin H-chain, which has ferroxidase activity. This form of storage is thought to protect iron from reduction (Balla *et al*, 1992). The L-chain has more iron storage capacity and facilitates iron core formation within the protein shell. The proportion of L and H chain depends on the tissue source as well as the level of iron loading. The mammalian H-Ft and L-Ft polypeptides show 50% homology and have the same ancestral gene (Ponka *et al*, 1998).

One Ft molecule could accommodate up to 4500 iron atoms as a crystalline core of ferric ions (Richter, 1978). Normally, Ft is only about 20% iron saturated (Reif, 1992) and as much as one third of Ft iron may turn over daily as a result of degradation and synthesis. Ft is slowly degraded to a non specific complex containing iron, which becomes a component of hemosiderin. Hemosiderin is a lipoprotein found in lysosomes, which happens to be a dead end in the metabolism of iron and Ft (Koorts and Viljoen, 2007). Ft has a high capacity to chelate iron by converting it to ferric state which is a safer form. As a result Ft is able to

restrict the Fe^{2+} availability to take part in Fenton reaction. Chelation of intracellular iron by storage in Ft, when the level of endogenous LI increases protects mammalian cells in culture against oxidative stress generated by a variety of factors (Balla *et al*, 1992; Vile *et al*, 1993 and 1994).

Despite Ft's iron scavenging property, it may also be a hazardous molecule, if exposed to pathological conditions leading to oxidative stress (see below).

1.5.6. Iron homeostasis and oxidative stress

Under pathological conditions, including oxidative stress, iron homeostasis is severely altered (Cairo and Pietrangelo, 2000; Deb *et al*, 2009). Disregulation of iron homeostasis leads an increase in intracellular LI, which may have harmful consequences on cells.

Iron bound to low molecular weight ligands constitutes a potential source of catalytic iron upon UVA radiation. *In vitro*, physiological doses of UVA radiation may reduce ferric iron bound to the potential low molecular weight intracellular chelator citrate (Vile and Tyrrell 1995).

Different conditions of stress such as UVA radiation (Aubailly *et al*, 1991), $\cdot\text{O}_2^-$ (Biemond *et al*, 1988) and reducing agents (Baader *et al*, 1994) have been shown *in vitro* to induce reductive release of iron from Ft.

Ft plays a dual role in LIP homeostasis. In one hand it acts as an iron-sequestering protein and in another hand act as a potential source of LIP. The cytoprotective role of Ft remained largely hypothetical until it was shown that cells overexpressing this protein are more resistant to oxidative injury (Balla *et al*, 1992; Cermak *et al*, 1993; Lin and Girotti, 1997). Overexpression of the H-subunit of Ft also decreased the LIP level (Picard *et al*, 1998) and

the H₂O₂-induced DNA damage (Cozzi *et al*, 2000). Conditional deletion of H-Ft in mice induced loss of iron storage and liver damage (Darshan *et al*, 2009). At cellular level, the deletion of H-Ft in mouse embryonic fibroblasts increased the intracellular LIP and probably as a direct consequence, ROS increased rapidly within the cells even at low extracellular iron concentrations (Darshan *et al*, 2009). Downregulation of H-Ft has also been shown to occur in murine and rat models of heart failure, leading to increase in LIP, oxidative stress and cell death (Omiya *et al*, 2009).

Various reductants and chelators, including physiological and toxicological substances, can also release iron from Ft (Koorts and Vilijoen, 2007). Effective reducing agents for the release of iron from Ft include flavins, cysteine, GSH, ascorbic acid and $\cdot\text{O}_2^-$ (Ponka *et al*, 1998).

Iron is also liberated from Ft as a consequence of normal turnover in lysosomal compartments, where it is recycled for heme synthesis (Vaisman *et al*, 1997; Radisky and Kaplan, 1998). This source of LIP has been shown to be active in the cell-damaging processes caused by oxidative stress promoting lysosomal rupture and release of potent hydrolytic enzymes to the cytosol (Ollinger and Brunk, 1995; Brunk *et al*, 1995, Roberg and Ollinger, 1998, Pourzand *et al*, 1999a).

It has been shown that during oxidative stress, iron could also be released from Ft as a result of its proteolytic degradation (Roberg and Ollinger, 1998, Pourzand *et al*, 1999a). For example studies from this laboratory have revealed that exposure of human skin fibroblasts to physiologically relevant doses of UVA, leads to immediate and dose-dependent increases in potentially harmful LI in skin cells that plays a key role in the increased susceptibility of skin cells to UVA-mediated oxidative membrane damage and necrotic cell death and almost certainly act to exacerbate damage caused by further exposure (Pourzand *et al*, 1999a; Zhong

et al, 2004; Reelfs *et al*, 2004). Further investigation in this field has revealed that UVA promotes immediate damage to both lysosomal and mitochondrial membranes. The lysosomal damage leads to leakage of potentially harmful lysosomal proteases into the cytosol which in turn attacks cytosolic proteins, notably the cytosolic iron storage protein, Ft. The proteolytic degradation of Ft contributes to an increase in cytosolic LI (see **Figure 11**).

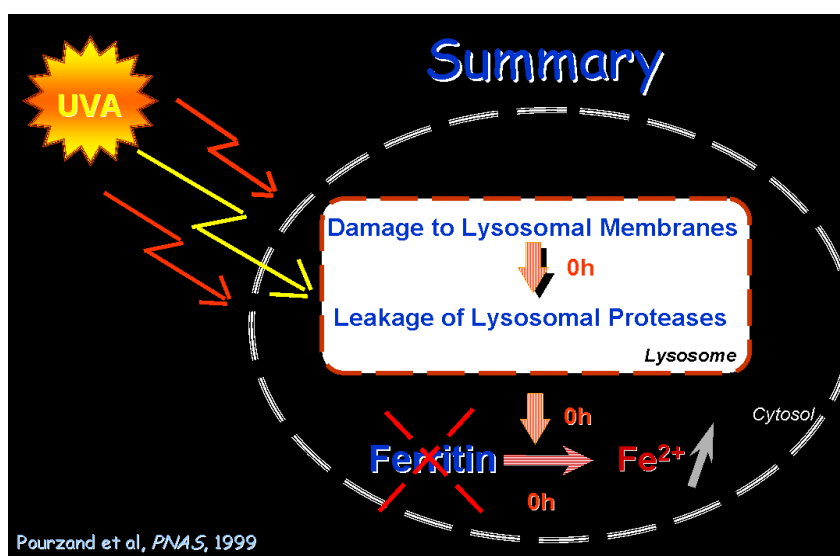


Figure 11. The UVA-induced damage to lysosomes. Exposure of skin fibroblasts to UVA promotes immediate (i.e. 0h) damage to lysosomal membrane leading to leakage lysosomal proteases into the cytosol. Once in cytosol, the lysosomal proteases degrade Ft and release its iron in the form of LI (Fe^{2+}) (Pourzand *et al*, 1999a).

The lack of cytosolic iron storage protein, Ft within the first hours after irradiation further exacerbate the iron-catalyzed damage in irradiated skin cells, since potentially harmful excess amounts of LI can not be safely sequestered. Indeed increase in intracellular LI along with ROS generated by UVA radiation promotes further peroxidative damage in exposed skin cells notably in plasma membrane that results in loss of cell membrane integrity. This

coincides with concomitant UVA-mediated damage to mitochondrial membrane that in turn leads to abrupt interruption of electron chain reactions within the mitochondrial membrane and production of ROS as well as depletion of mitochondrial ATP, all of which lead to necrotic cell death (Zhong *et al*, 2004) (see **Figure 12**).

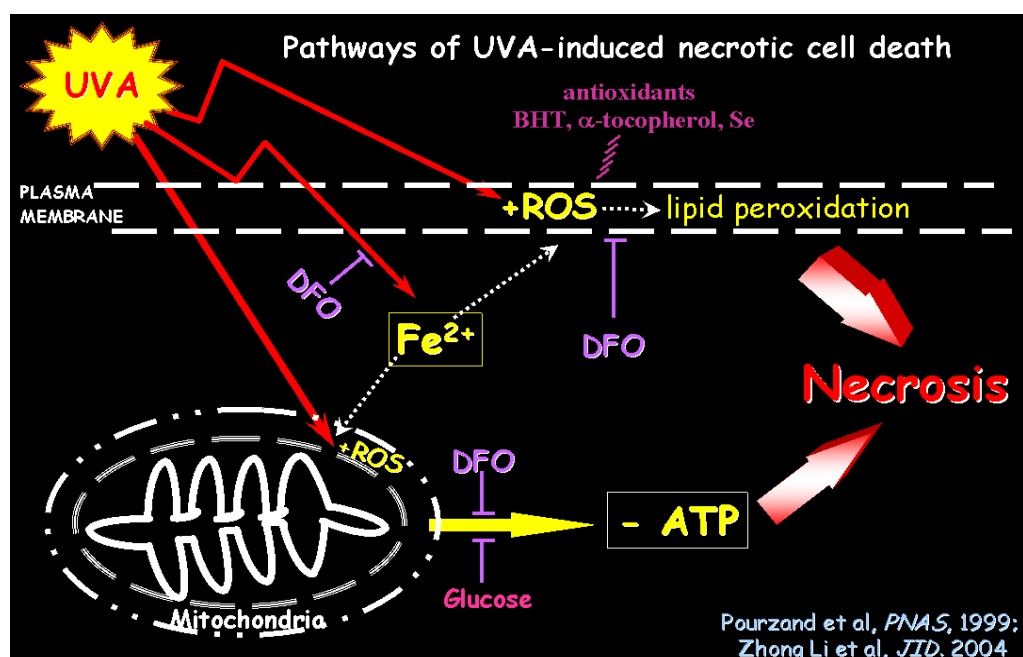


Figure 12. Pathways of UVA-induced necrotic cell death in skin fibroblasts. Exposure of skin fibroblasts to UVA promotes immediate (i.e. 0h) release of labile iron (Fe^{2+}) that exacerbate the peroxidative damage in plasma and mitochondrial membranes leading to loss of intracellular ATP and loss of membrane integrity, both of which cause necrotic cell death. Pretreatment of cells with strong iron chelators such as desferal (DFO) or membrane antioxidants (e.g selenium; se, butyryl hydroxytoluene ; BHT or α -tocopherol) could protect the cells against UVA-induced membrane damage and necrosis. Restoring the intracellular ATP with glucose could protect the cells from necrosis (Pourzand *et al*, 1999a; Zhong *et al*, 2004; Reelfs *et al*, 2010).

The potential role of Ft iron in exacerbating UVA-induced damage was further strengthened by studies showing that increasing Ft synthesis in cells by iron loading in the form of iron citrate or hemin, could lead to substantial increase in UVA-induced LI release and necrotic cell death (Zhong *et al*, 2004; Reelfs *et al*, 2004; Reelfs *et al*, 2010). Conversely conditional deletion of H-Ft in an oxidant-sensitive strain of mouse embryo fibroblasts (Darshan *et al*, 2009) caused a decrease in UVA-induced iron release and cell damage (unpublished data, Pourzand's laboratory- personal communication)

Oxidative stress can also affect iron homeostasis via activation of the haem-catabolising enzyme HO-1 as exemplified in UVA-irradiated skin fibroblasts cells. Studies from Tyrrell's laboratory have shown that UVA promotes haem release from microsomal haemoproteins immediately after UVA irradiation of FEK4 cells (Kvam *et al*, 1999). Although haem itself is not a source of LIP, it is a substrate for HO-1 and HO-2, both of which could release the haem iron and as such contribute to the increase in potentially harmful LIP observed after UVA irradiation. Indeed Kvam *et al* (2000) have shown that HO-activity can cause hypersensitivity to oxidative UVA radiation due to release of iron from haem. The UVA-mediated transcriptional activation of HO-1 gene eventually (*i.e.* 1-2 days) leads to HO-1-dependent increase in Ft (Vile and Tyrrell, 1993) and a consequent lowering of the pro-oxidant state in skin cells (Vile *et al*, 1994).

Iron regulatory protein-1 (IRP1), which may itself act as potentially important iron/citrate carrier, is susceptible to oxidative inactivation of RNA binding *in vitro* and *in vivo* (Hentze *et al*, 1989; Mullner *et al*, 1992; Cairo *et al*, 1996). Down-regulation of IRP1 occurs also *in vivo* in liver tissue of the rats subjected to ischemia reperfusion or phorone, a GSH-depleting agent (Cairo *et al*, 1995; Tacchini *et al*, 1997). Exposure of skin cells to UVA also inactivates IRP1

binding activity (Pourzand *et al*, 1999b). By contrast, menadione, nitric oxide, and H₂O₂ (Martins *et al*, 1995; Pantopoulos and Hentze, 1995) activate IRP1 RNA binding activity and, as a result, lead to a coordinated decrease of Ft synthesis and induction of TfR expression. Nevertheless exposure to H₂O₂ has been shown to induce LI release, although its underlying mechanism is under debate (Breuer *et al*, 1997; Yiakouvaki *et al*, 2006).

1.6. Photodynamic therapy (PDT)

1.6.1. Definition

The combination of photosensitising chemicals and light to treat skin diseases has been widely practiced in dermatology for centuries. This practice dates back to ancient Egypt, India and Greece, where psoralen-containing plants extracts and light were applied to treat psoriasis, vitiligo and skin cancer (Wilson and Mang, 1995). This concept, generally known as photochemotherapy, represents a common basis for different treatment procedures such as PUVA (i.e. psoralen-UVA) and photodynamic therapy (PDT).

Photochemotherapy is a form of phototherapy in which a chemical substance is used in addition to light. In PDT, O₂, light and a photosensitising drug are used in combination (see **Figure 13**). The first component of PDT is the ‘photosensitiser’ – a photosensitive molecule that localizes to a target cell and/or tissue. The second component involves the administration of light of a specific wavelength that activates the photosensitiser. The photosensitiser transfers energy from light to molecular oxygen, to generate ROS (Ryter and Tyrrell, 1998). These reactions occur in the immediate locality of the light-absorbing photosensitiser. Therefore the biological responses to the photosensitiser are activated only in the particular areas of tissue that have been exposed to light.

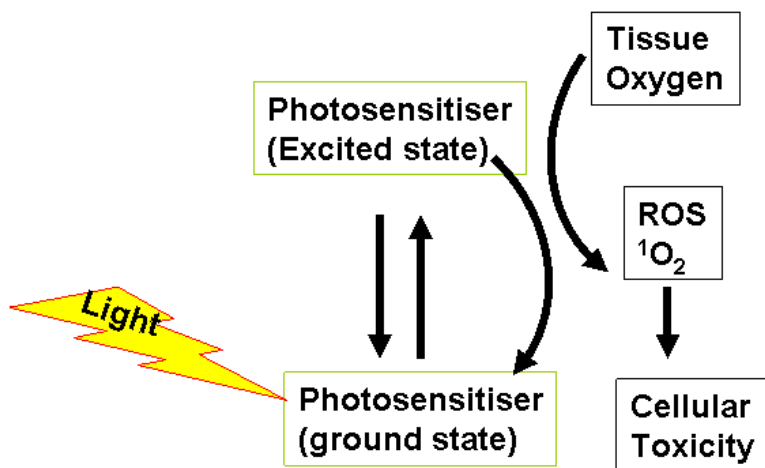


Figure 13. Mechanism of action of photodynamic therapy (PDT). PDT requires three elements: light, a photosensitiser and oxygen. When the photosensitiser is exposed to specific wavelengths of light, it becomes activated from a ground to an excited state. As it returns to the ground state, it releases energy, which is transferred to oxygen to generate ROS, such as singlet oxygen ($^1\text{O}_2$) and free radicals. The generated ROS mediate cellular toxicity.

1.6.2. Cell/tissue damage mechanisms of PDT

PDT is a selective treatment modality that affects mainly the target tissue. The selectivity is based on a difference in the photosensitiser concentration between normal and target tissues and on the directing light into the target tissue. One advantage of PDT is that the photosensitiser can be administered orally, intravenously or by topical application to the skin. However these affect its biodistribution (Dolmans *et al*, 2003). Because biodistribution changes over time, the timing of light exposure is another way to regulate the effects of PDT. After administration, the photosensitiser accumulates in the target tissue during 3-96 h, depending on the photosensitiser used. After this accumulation period, light directed to the tissue activates the photosensitiser and, in the presence of ground-state (triplet) oxygen ($^3\text{O}_2$),

ROS are formed. Briefly, following the absorption of light (photons), the sensitiser is transformed from its ground state (single state) into a relatively long-lived electronically excited state (triplet state) *via* a short-lived excited singlet state (Henderson and Dougherty, 1992). The excited triplet can undergo two kinds of reactions (see **Figure 14**). First it can react directly with a substrate such as the cell membrane or a molecule and transfer hydrogen atom (electron) to form radicals. These radicals in turn can transfer their excessive energy to ground state oxygen ($^3\text{O}_2$) to form $^1\text{O}_2$ and other oxygenated reactive species (**type I reaction**). Alternatively, the triplet can transfer its energy directly to ground state oxygen, to form $^1\text{O}_2$ (**type II reaction**).

Both type I and type II reactions occur simultaneously and the ratio between these processes depends on the type of photosensitiser used, the concentrations of substrate and oxygen, as well as the binding activity of the photosensitiser for the substrate. Because of the high reactivity and short half-life of the ROS, only cells/ cellular components that are proximal to the area of the ROS production (*i.e.* areas of photosensitiser localisation) are directly affected by PDT (Moan and Berg, 1991). Among generated ROS, $^1\text{O}_2$ is regarded as the main mediator of phototoxicity in PDT as it is a powerful oxidant that can react with many kinds of biomolecules. These include unsaturated triacyl glycerols, cholesterol, phospholipids, amino acids such as tryptophan, histidine and methionine as well as nucleic acid bases such as guanine and guanosine (Milgrom and MacRobert, 1998; Stenberg *et al*, 1998). The generated ROS then damage vital structures and functions of cells, which result in tissue destruction (Bonnet, 1999; Milgrom and MacRoberts, 1998; Nyman and Hynninen, 2004). Eventually, however, the photosensitiser is degraded by light. This process, known as ‘photobleaching’ can results from reactions of type I or type II (see also **section 1.3.1.3 and 1.3.2**). Photosensitized, O_2 -dependent reactions in biological systems are known as photodynamic action (Spikes, 1997).

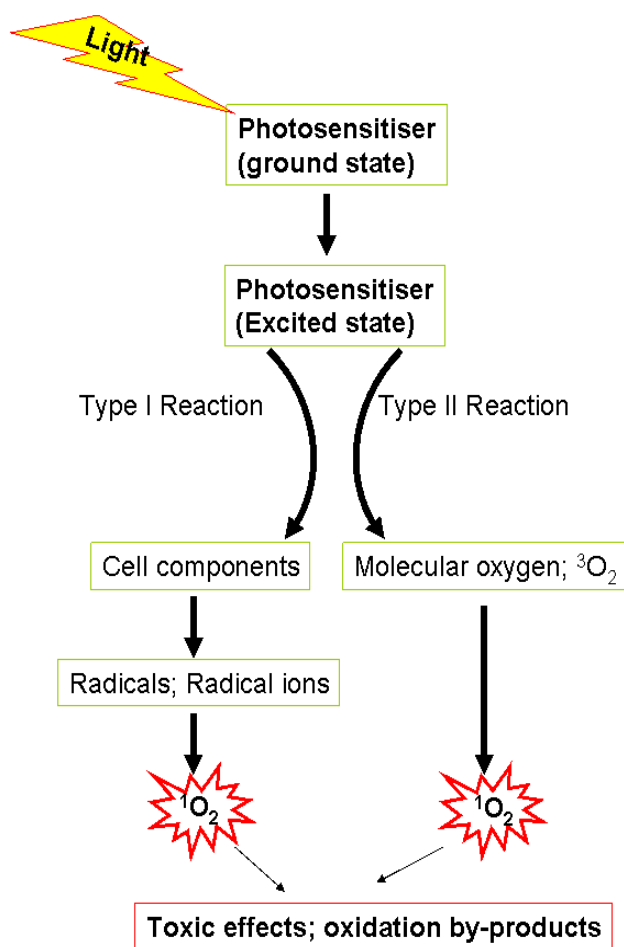


Figure 14. Type I and Type II reactions in PDT. Following the absorption of light, the sensitizer is transformed from its ground state into an excited state. The activated sensitizer can undergo two kinds of reaction. First it can react directly either with the substrate such as the cell membrane or a molecule, transferring a hydrogen atom to form radicals. The radicals interact with oxygen to produce oxygenated product ($^1\text{O}_2$) (type I reaction). Alternatively the activated sensitizer can transfer its energy directly to oxygen to form $^1\text{O}_2$ (type II reactions). These species oxidize various substrates and damage vital structures and functions of cells.

The extent of photodamage and cytotoxicity is multifactorial and depends on the type of photosensitizer, its extracellular and intracellular localization, the total dose administered, the total light exposure dose, light fluence rate, oxygen availability and the time between the administration of the drug and light exposure. All of these factors are interdependent. (Palumbo, 2007; Dolmans *et al*, 2003; Nowis *et al*, 2005).

It is generally acknowledged that PDT achieves tumour damage by three interdependent processes (i) direct tumour cell killing; (ii) damage to the vasculature and (iii) activation of non specific immune response (Golab *et al*, 2000; van Duijnhoven *et al*, 2003; Abels, 2004). Direct cell killing is the most important effect seen after photoactivation that appears to also be photosensitiser-dependent (Henderson *et al*, 1985). Cell death is brought about by the local formation of ROS. However both the non-homogenous distribution of oxygen within the tumour that may lead to non-homogenous activation of the photosensitiser and the asymmetrical distribution of the photosensitiser within the tumour mass and surrounding tissue could result in incomplete therapeutic response (Korbelik and krosi, 1996). Oxygen shortage can arise as a result of the photochemical consumption of oxygen during the photodynamic process as well as from the immediate effects of PDT on the tissue vasculature. Indeed rapid and substantial reduction in the tissue oxygen tension during and after illumination of photosensitised tissue has been reported (Tromberg *et al*, 1990; Pogue *et al*, 2001). Although the development of microvascular damage and hypoxia after PDT has been shown to contribute to the long-term tumour response, the reduction in oxygen that occurs during PDT can limit the response. There are two ways to overcome this problem. One is to lower light fluence rate to reduce oxygen consumption rate and the other is to fractionate the PDT light delivery to allow re-oxygenation of the tissue (Messman *et al*, 1995; Pogue and Hasan, 1997; Curnow *et al* 2000).

Cell death in PDT may occur by apoptosis or necrosis, depending on the sensitiser, PDT drug-light dose and on the cell genotype (Almeida *et al*, 2004). Apoptosis is a tightly controlled, energy-consuming process of suicidal cell death involving activation of hydrolytic

enzymes such as proteases and nucleases leading to DNA fragmentation and degradation of intracellular structures (Reed, 2000). Necrosis is the pathological process, which occurs when cells are exposed to a serious of physical or chemical insult whereas apoptosis or programmed cell death is the physiological process responsible for the elimination of superfluous, aged or damaged cells.

Although apoptosis is necessary for both health and disease, necrosis is always the outcome of severe and acute injury. Apoptosis and necrosis have long been considered as two distinct mechanisms of cell death, each with different biochemical and morphological characteristics (Wyllie *et al*, 1980; Majno and Jorris, 1995). Apoptotic cells are defined by fragmented nuclei with condensed chromatin, fragmented or condensed cytoplasm and formation of apoptotic bodies, whereas necrotic cells are characterized by electron-lucent cytoplasm, mitochondrial swelling and loss of plasma integrity without severe nuclear damage (see **Figure 15**).

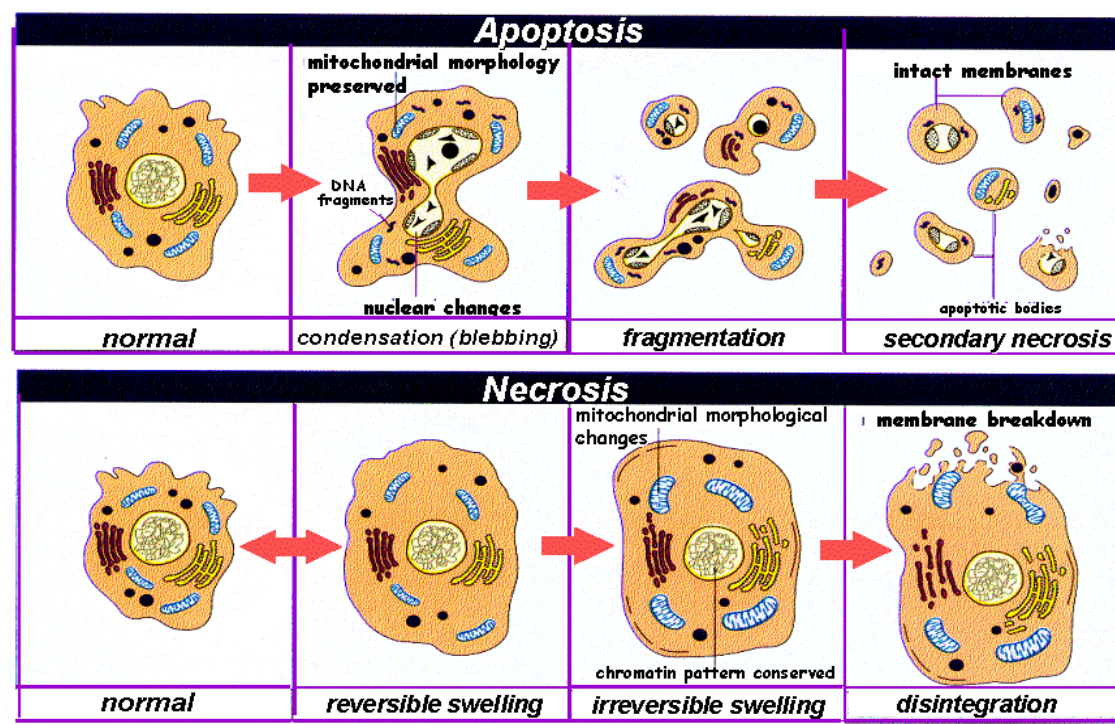


Figure 15. A comparison of different morphological features of apoptosis versus necrosis. In apoptotic cell death plasma membrane is intact until late stage; however cytoplasm shrinks and nucleus condenses in early stage of apoptosis. Cells then fragment into smaller bodies called apoptotic bodies followed by lysis in a process called ‘secondary necrosis’. In necrotic cell death, early loss of membrane integrity leads to swelling of cytoplasm and mitochondria. This in turn leads to cell membrane breakdown and disintegration of organelles (i.e. complete lysis). Adapted from ‘Apoptosis and cell proliferation’, second edition, Roche.

It has been shown that both necrosis and apoptosis are rapid and dominant form of cell death following PDT in multiple experimental settings utilizing various photosensitisers and cell types (Oleinick *et al*, 2002). Indeed, increasing amount of experimental data indicate that at optimal PDT conditions (i.e. sufficient photosensitiser concentration and light exposure) tumour cells die by necrosis (Nowis *et al*, 2005). Factors that promote necrosis may include

extra-mitochondrial localization of photosensitiser, high dose PDT and glucose starvation (Almeida *et al*, 2004; Dellinger, 1996; Kiesslich *et al*, 2005; Oberdanner *et al*, 2002; Kirveliėne *et al*, 2003; Plaetzer *et al*, 2002), although the research in this field is still ongoing.

Apoptotic cell death in PDT is mostly observed with photosensitisers that accumulate in mitochondria (Krieg *et al*, 2003; Fabris *et al*, 2001). However increasing the photosensitiser concentration and light dose usually switches the apoptotic cell death to necrosis. The latter appears to be related to the availability of intracellular ATP following PDT. Indeed it is now well established that the progression to necrotic or apoptotic cell killing following death stimuli depends on the effect the mitochondrial membrane damage has on cellular ATP levels (Reed *et al*, 1998). Damage to mitochondrial membranes can trigger both the abrupt opening of a high conductance permeability transition pore in the mitochondrial inner membrane and cytochrome c release from these organelles, both of which could lead to interruption of electron chain transport causing the generation of ROS, loss of the electrochemical gradient across the inner membrane and ATP depletion followed by necrosis (Reed, 1999 and Reed *et al*, 1998) (see **Figure 16**). As the presence of ATP is essential for the activation of apoptosis protease activating factor-1 (Apaf-1) and subsequent activation of caspases that induce apoptosis (Pourzand and Tyrrell, 1999), it appears that the high PDT dose insult (i.e. causing ATP depletion as a result of severe damage to mitochondrial membrane) as well as glucose starvation (i.e. interrupting the main supply of intracellular ATP) could trigger predominantly necrotic cell death in treated cells/tissues. Therefore photochemical damage of cellular targets involved in energy supply especially mitochondria might play a crucial role in the mode of cell death.

For example Plaetzer and coworkers (2002) have demonstrated that when human epidermoid carcinoma cell A431 is treated with a phthalocyanine-based photosensitiser, the intensity of the light dose employed could determine the mode of cell death after PDT depending on the changes occurring in mitochondrial function and intracellular ATP. They observed that at moderate doses of 2.5-3.5 J/cm², 50% of cells died by either apoptosis or necrosis. However at a higher light dose of 6 J/cm², neither caspase 3 activation nor nuclear fragmentation was observed and cells died exclusively by necrosis. Necrotic cell death was associated with a rapid decline in mitochondrial activity and intracellular ATP. By contrast with apoptosis, the loss of mitochondrial function was delayed and the ATP level was maintained at near control levels. This study suggests that the magnitude of light dose rather than the photosensitiser plays a critical role in the decision of the cell to undergo either apoptotic or necrotic cell death. Therefore apoptosis and necrosis may not necessarily be two independent pathways but may share common events, at least in early phases of cell death process. This assumption is strengthened by a series of studies showing that the magnitude of initial insult of various oxidants could be the main determinant of apoptotic and/or necrotic cell death processes (e.g. Bonofoco *et al*, 1995; Hampton *et al*, 1997).

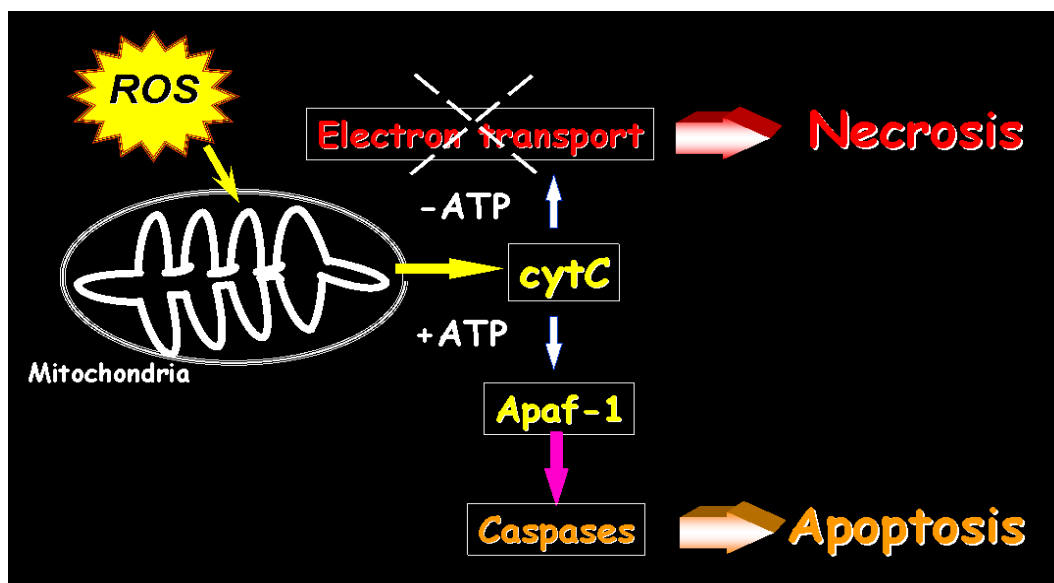


Figure 16. Mitochondrial damage can induce both apoptotic and necrotic cell death. The first step in the induction of cellular death by various generators of ROS (e.g. PDT and oxidative stress) is the increase in mitochondrial membrane permeability and the opening of the mitochondrial permeability transition pore that leads to collapse of mitochondrial membrane potential. As a consequence of mitochondrial dysfunction, cytochrome C (cyt C) is released from the mitochondrial intermembrane space in to the cytosol which acts in concert with ATP to induce conformational change in Apaf-1 (apoptotic protease activating factor-1). The conformational change of Apaf-1 allows it to bind to pro-caspase 9 and form the apoptosome complex. The apoptosome will then act as the initiator of downstream caspase cascade activation. However severe damage to mitochondrial membrane can trigger the abrupt opening of a high conductance permeability transition pore in the mitochondrial inner membrane and cyt C release from these organelles, both of which could lead to interruption of electron chain transport causing the generation of ROS, loss of the electrochemical gradient across the inner membrane and ATP depletion followed by necrosis. (see Reed *et al*, 1999). (Reproduced from Dr Pourzand's slides with permission) (Zhong *et al*, 2004).

Studies aimed at investigating the pathways of apoptotic cell death induced by the non-membrane damaging agent staurosporine have already established the importance of ATP in the switch between apoptosis and necrosis. Indeed, depletion of ATP by compounds such as oligomycin switched the apoptotic cell death to necrosis (e.g. Eguchi *et al*, 1997; Leist and

Nicotera, 1997; Ha and Snyder, 1999), whereas repletion of ATP by glucose or fructose (*via* glycolysis) in the oligomycin treated cells was able to restore the apoptotic cell death.

An interesting study by Kirveliène and coworkers (2003) demonstrated that intracellular ATP concentration correlates with the mode of cell death initiated in murine hepatoma MH22 cells *in vitro* by photosensitisers of mitochondrial but not lysosomal localization: (i) cells photosensitised with meso-tetra(4-sulfonatophenyl)-porphine (TPPS4; **see section 1.6.4.3**) localized to lysosomes (Boyle and Dolphin, 1996) died mostly by necrosis, and the mode of cell death did not depend on the energy metabolism; (ii) cells photosensitised with 5,10,15,20-tetrakis(m-hydroxyphenyl)-chlorine (m-THPC, **see section 1.6.4.2**) localized to cell membranes including mitochondria (Boyle and Dolphin, 1996), or aminolaevulinic acid (ALA, **see section 1.6.**)-induced endogenous porphyrins predominantly localized in mitochondria, under conditions of favorable to glycolysis led to cell death mostly by apoptosis, however under conditions unfavorable to glycolysis (*i.e.* ATP depletion), led to considerable decrease in the ratio of apoptosis/necrosis. So in m-THPC- and ALA-mediated PDT, intracellular ATP and caspase activation appear to be the downstream controllers capable of directing the cells toward either type of cell death *i.e.* under conditions favorable to glycolysis, the intracellular ATP is highly maintained so the PDT insult favours apoptosis over necrosis whereas ATP depletion under conditions unfavorable to glycolysis transforms apoptosis to necrosis due to inhibition of caspase activation.

Nevertheless, in general in PDT, during illumination the concentration of the photosensitiser may decrease due to photobleaching, vasculature stasis, tumour oedema etc. In these conditions, as well as in deep tumour regions (illuminated with suboptimal light from the very beginning of treatment), the effectiveness of PDT is decreased and tumour cells have a

chance to either oppose or repair damage induced by the treatment. If the damage is repaired, they will survive the treatment and will contribute to tumour relapse (if not damaged by lethal ischaemia or activation of immune response) (Nowis *et al*, 2005). In all other cases tumour cells should undergo apoptosis or necrosis depending on the severity of the PDT insult and ATP depletion. However if the damage remains unrepaired, this will lead to mutagenesis.

1.6.3. First generation of photosensitisers

The term ‘photodynamic therapy’ was first described in 1903 for oxygen-consuming chemical reactions induced by photosensitization in biology (Dougherty *et al*, 1998). The use of porphyrins, the photosensitiser with which the majority of clinical studies have been conducted in photodynamic therapy, was introduced in 1961 by Lipson and coworkers. During the period from 1961 to 1983, Haematoporphyrin (HP) and its derivatives were the most commonly used photosensitiser in PDT research (Bonnett, 1999). Hence these tetrapyrroles comprise the first generation of photosensitisers.

In 1960, Lipson and Baldes attempted to purify and solubilise HP by treatment with 5% sulfuric acid in acetic acid, basification and neutralization to pH 7.4 with hydrochloric acid. The purified compound, named hematoporphyrin derivative (HPD) had a better affinity for tumor tissue than crude HP and could be visualized when injected into the tumour by fluorescence endoscopy (Lipson and Baldes, 1961). Early studies very rapidly expanded to investigate the phototherapeutic potentials of HPD in preclinical and clinical studies in the 1970s. In 1972, Diamond and colleagues postulated that the combination of the tumour-localizing and tumour-phototoxic properties of porphyrins might be exploited to kill cancer

cells (Diamond *et al*, 1972). In 1978, Dougherty and coworkers, demonstrated that parenteral HPD administration could be activated by light in the red region of the spectrum (630 nm) to cause complete eradication of spontaneous or transplanted mammary tumours in mice and rats (Dougherty *et al*, 1978). Within the same period, Kelly and co-workers reported that light activation of HPD also eliminated bladder carcinoma in mice (Kelly *et al*, 1975). These studies classified HPD as the benchmark photosensitiser for the development of forthcoming drugs (Kalka *et al*, 2000; Dolmans *et al*, 2003).

HPD is a very complex mixture of several components, of which approximately 50% is identifiable as oligomeric haematoporphyrins and protoporphyrins. A further chemical purification allows for the preparation of porfimer sodium (Photofrin ®), which is a lyophilised and concentrated form of monomeric (haematoporphyrin, protoporphyrin and monohydroxyethyl-vinyl-deuteroporphyrin) and oligomeric (dimer to hexamer derivatives of hematoporphyrin units linked through ether or ester bonds) porphyrins (see **Figure 17**) and reduced fraction of components that are not photoactive. The purified compound Photofrin has shown enhanced photosensitising properties (Lui, 1992; Dougherty, 1987). Photofrin was the first approved photosensitising drug for clinical PDT of lung, oesophageal, gastric and cervical cancer (Evensen, 1995).

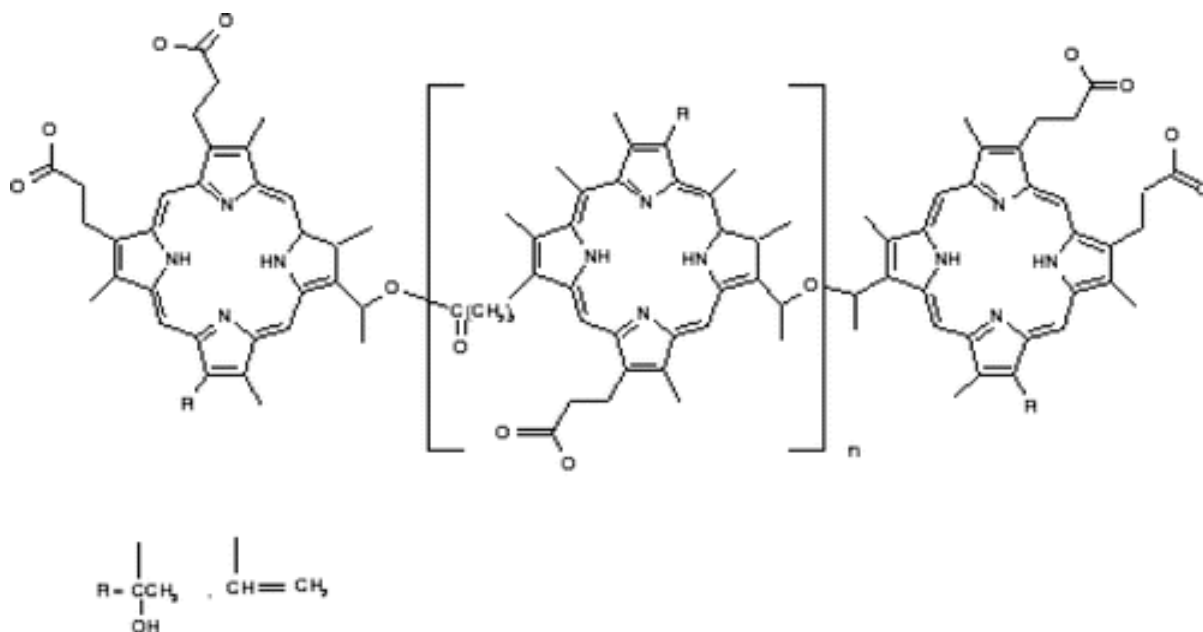


Figure 17. The chemical structure of porfimer sodium, a complex mixture of monomers and oligomers (n=2-6).

The intravenous administration of Photofrin or HPD leads to maximal tumour-to-normal cell concentration ratios 24 to 48h after treatment. The absorption spectrum of porphyrins exhibits a maximum in the Soret band ranging from 360 to 410 nm, followed by 4 smaller peaks between 500 nm and 635 nm (Q bands) (see **Figure 18**). Unfortunately, significant tissue penetration is achieved by light at 630 nm to 635 nm, which corresponds to the weakest of the absorption peaks (Stables and Ash, 1995) allowing for a penetration of about 5-10 mm in therapeutic PDT (Nyman and Hynninen, 2004).

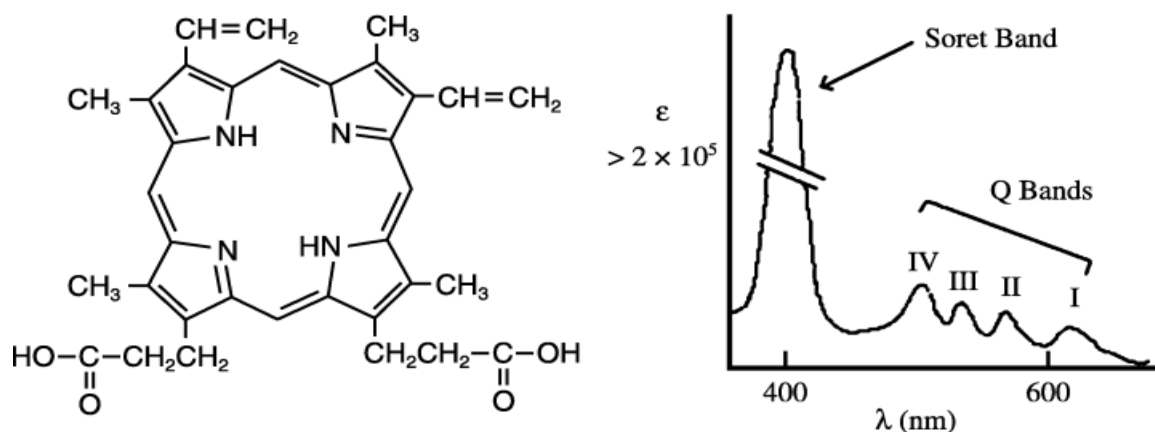


Figure 18. The absorption spectrum of porphyrins (right) as exemplified by protoporphyrin IX (the chemical structure; left).

The cutaneous accumulation of first generation porphyrin-based photosensitising drugs and their slow clearance from the skin leads to long-lasting cutaneous photosensitivity, requiring photoprotective measures during 4-6 weeks after PDT (Gomer, 1991). An approach to avoid this side effect is the use of topical formulations (e.g. Steiner *et al*, 1995). However the number of reports is very limited and major studies using PDT with topical first generation photosensitisers are lacking (Kalka *et al*, 2000).

The poor selectivity of first generation photosensitisers between tumour and normal tissue and their long clearance time that led to prolonged skin photosensitivity in subjects encouraged the investigations to design improved synthetic photosensitisers for PDT. These compounds are referred to as 'second generation photosensitisers'.

1.6.4. Second generation of photosensitisers

Following the initial successful attempts to treat specific cancers with PDT, a number of second generation photosensitisers have been developed. In contrast to Photofrin and HPD which are mixtures, these drugs are pure substances and present a higher absorbance in the far red (660-700 nm) or near infrared (700-850 nm) regions of the spectrum. The higher molar absorption coefficient results in a more intense excitation of the photosensitiser at a wavelength that also allows a deeper penetration into tissues (up to 20 mm compared to 5-10 mm at 630 nm) and consequently a more efficient therapeutic effect (provided that sufficient oxygen is available) (Nyman and Hynninen, 2004; Wilson *et al*, 1984). In addition, near-infrared absorbing photosensitisers should allow for the treatment of highly pigmented tumours such as melanoma metastases, which are unaffected by photosensitisers that absorb in the visible range. Light with a wavelength longer than 850 nm is not used because it does not yield enough energy to trigger a photochemical reaction (Calzavara-Pinton *et al*, 2007).

The second generation of photosensitisers includes many families of molecules such as modified porphyrins, bacteriochlorins, phthalocyanines, naphthalocyanines, pheophorbides and purpurins. Most of these photosensitisers are used as PDT agents in clinical trials (Stenberg *et al*, 1998) for various cancers, including cutaneous malignancies. A brief description of these photosensitisers is provided below. **Figure 19** illustrates the chemical structure of some of these porphyrin derivatives.

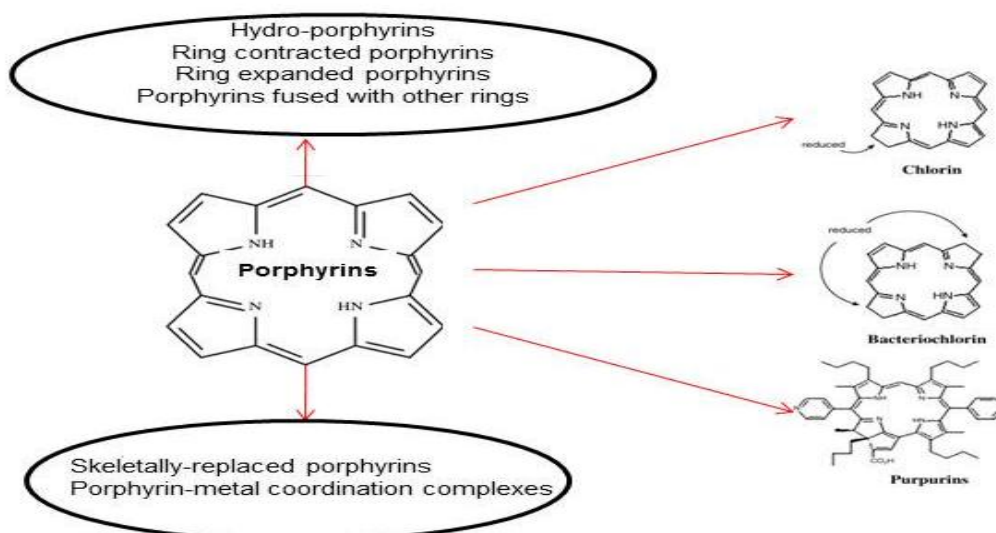


Figure 19. Chemical structure of some of porphyrin-derived sensitisers (Modified from Calzavara-Pinton *et al*, 2007).

1.6.4.1. Phthalocyanines:

The joining of four benzene or naphthalene rings to the α -pyrrolic position of porphyrins and substituting the methane bridge carbons with nitrogen produces phthalocyanines and naphthalocyanines (see **Figure 20**). Because these compounds absorb long wavelength light strongly, they can be used at much smaller doses than Photofrin (*i.e.* at a dose 4-5 fold lower than Photofrin). Furthermore as phthalocyanines and naphthalocyanines do not strongly absorb light in the 400-600 nm range, they therefore provide a lower risk of generalised photosensitivity to sunlight than porphyrins (Moan and Berg, 1992; Jori, 1992).

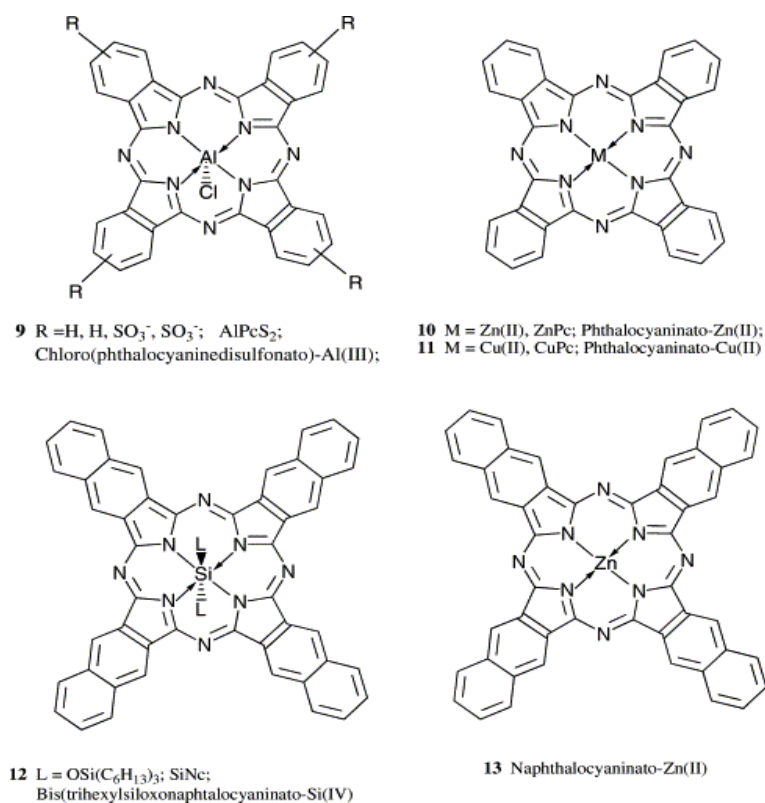


Figure 20. Chemical structure of phthalocyanines and naphthalocyanines that are relevant to PDT. (source: Nyman and Hynninen, 2004).

Phthalocyanines show promises for PDT of cancer. The incorporation of diamagnetic metals such as Zn or Al into the phthalocyanine macromolecule increases the half-life of triplet state and guarantees a satisfactory yield of ¹O₂ generation for efficient photosensitisation and phototoxicity in the target tissue (Jori, 1992). Examples include the phthalocyanine derivative AlPcS₂ (chloroaluminium sulphonated phthalocyanine). These compounds have higher chemical stability, superior direct tumour cell phototoxicity and a strong absorption peak in the red spectrum (650-700 nm), that provide deeper penetration into the tissue as compared with porphyrins (Spikes, 1986; Oleinick *et al*, 1993; Rosenthal, 1991). These properties coupled with negligible dark toxicity, and excellent photodynamic activity at increased

wavelengths has led to the clinical evaluation of phthalocyanines notably ALPcS2 for PDT (Ben-Hur, 1992; Gerber-Leszczynszyn *et al*, 2004). Nevertheless the slow rate of clearance of ALPcS2 may cause the problem of photosensitivity for topical applications of the compound for skin cancer lesions (Peng and Moan, 1995).

1.6.4.2 Chlorin and Bacteriochlorin derivatives:

The reduction of a peripheral double bond of porphyrin strengthens the longest wavelength band and moves the absorption peak to the red. The dihydroporphyrins thus obtained are called ‘chlorins’ (Nyman and Hynninen, 2004) (see **Figures 19** and **21**). Among chlorin derivatives, the meta-tetrahydroxyphenyl chlorine (m-THPC) was originally introduced in clinical trials for the treatment of mesothelioma and is now widely used in many countries to treat respiratory, gynaecological and head and neck cancers (Hopper, 2000 and **Figure 19**). The photosensitiser m-THPC (Temoprofin, Foscan®; Biolitech Pharma, Edinburgh, Scotland, UK) is a pure compound whose effectiveness in PDT has been reported as a 200-fold higher than Photofrin (Sharman *et al*, 1999). Compared to Photofrin, it is excited at a longer wavelength (i.e. 650 rather than 630 nm) and has a higher molar absorbance coefficient (i.e. 15-fold higher). It has also a longer half-life than Photofrin in the excited state and its higher hydrophobicity facilitates cellular uptake and as a result its use for clinical indications has been extensively investigated (e.g. Copper *et al*, 2003) for head and neck SCC and (e.g. Campbell *et al*, 2004) to target intraepithelial lesions. The comparative *in vitro* studies on the dark and light-dependent cytotoxic effects of m-THPC formulations with other second generation photosensitisers has proven the reputation of this chlorin as very effective photosensitiser. Fospeg®, a water soluble formulation of m-THPC, shows a strongly reduced dark toxicity and a similar phototoxic efficiency compared to Foscan®. Foscan® and

Fospeg® induce apoptosis in epidermoid carcinoma cells, A431 over a relatively wide range of fluences (Allison *et al*, 2004; Berlanda *et al*, 2010). Due to their efficacy and absorption at 654 nm they could be both suitable for treatment of malignancies characterised by a greater extension; for example a tumouricidal depth of up to 8 mm has been demonstrated for pancreatic cancer (Bown *et al*, 2002). Nevertheless the residual photosensitivity of m-THPC is comparable to that of Photofrin (Palumbo, 2007).

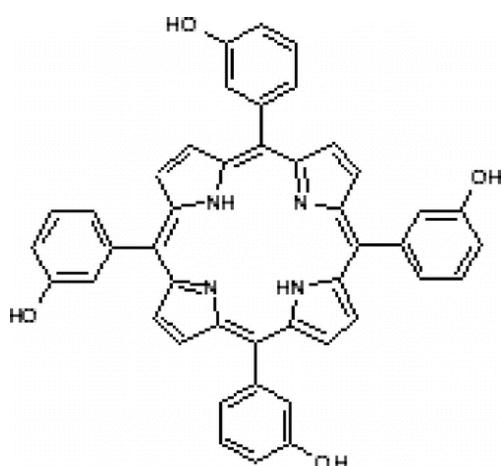


Figure 21. Chemical structure of meta-tetrahydroxyphenyl chlorine (m-THPC)

Other chlorin derivatives such as mono-L-aspartyl chlorin e6 (NPe6, Porphyrin Products Inc, Logan UT, USA) was reported to have high effectiveness in clinical trials (e.g. Wong *et al*, 2003) (see **Figure 22**). Although this compound provokes only a very mild and transient photosensitivity, for adequate tumour response it requires high photosensitiser concentration that results in a decrease in tissue selectivity. Both NPe6 and chlorin e6 (Dialek LTd, Minsk, Belarus) are hydrophilic compounds with similar photobiological properties. They have been investigated as photosensitisers in dermatology for the PDT of various cutaneous and subcutaneous malignancies, including recurrent adenocarcinoma of the breast as well as basal

cell carcinoma (BCC) and squamous cell carcinoma (SCC) (*e.g.* Taber *et al*, 1998; Gomer and Ferrario, 1990; Calzavara-Pinton *et al*, 1998; Sheleg *et al*, 2004).

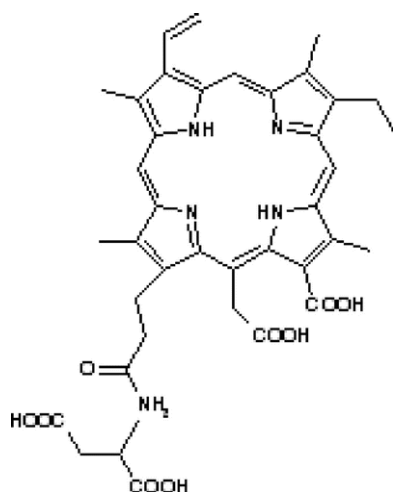


Figure 22. Chemical structure of mono-aspartyl chlorin e6 (NPe6)

Purpurins contain the porphyrin macrocycle and have absorption bands of 630-715 nm (see **Figure 23**). Among purpurins, Tin etiopurpurin (SnET₂) is a synthetic chlorin analogue with maximal excitation at 660 nm (Wilson and Mang, 1995). It is a mild photosensitiser and it takes few days after the treatment to clear from the skin, although cutaneous reactions occurring one or more months after PDT have been reported (Morgan *et al*, 1988; Garbo *et al*, 1996). Nevertheless SnET₂ has been used successfully for the photodynamic management of BCC, Bowen's disease (BD), cutaneous metastatic breast cancer and AIDS-related Kaposi sarcoma (*e.g.* Bissonnette and Lui, 1997; Wilson and Mang, 1995).

A number of companies are also developing promising bacteriochlorins with improved optical properties absorbing light at more than 740 nm that might be particularly useful for PDT of pigmented tumours (Jori, 1992 and **Figure 23**). Among these compounds, SQN400

(trade name for meta-tetra-hydroxyphenyl bacteriochlorin; mTHPBC) has recently been used in some clinical Phase I studies (*e.g.* van Duijnhoven *et al.*, 2005) (see **Figure 24**).

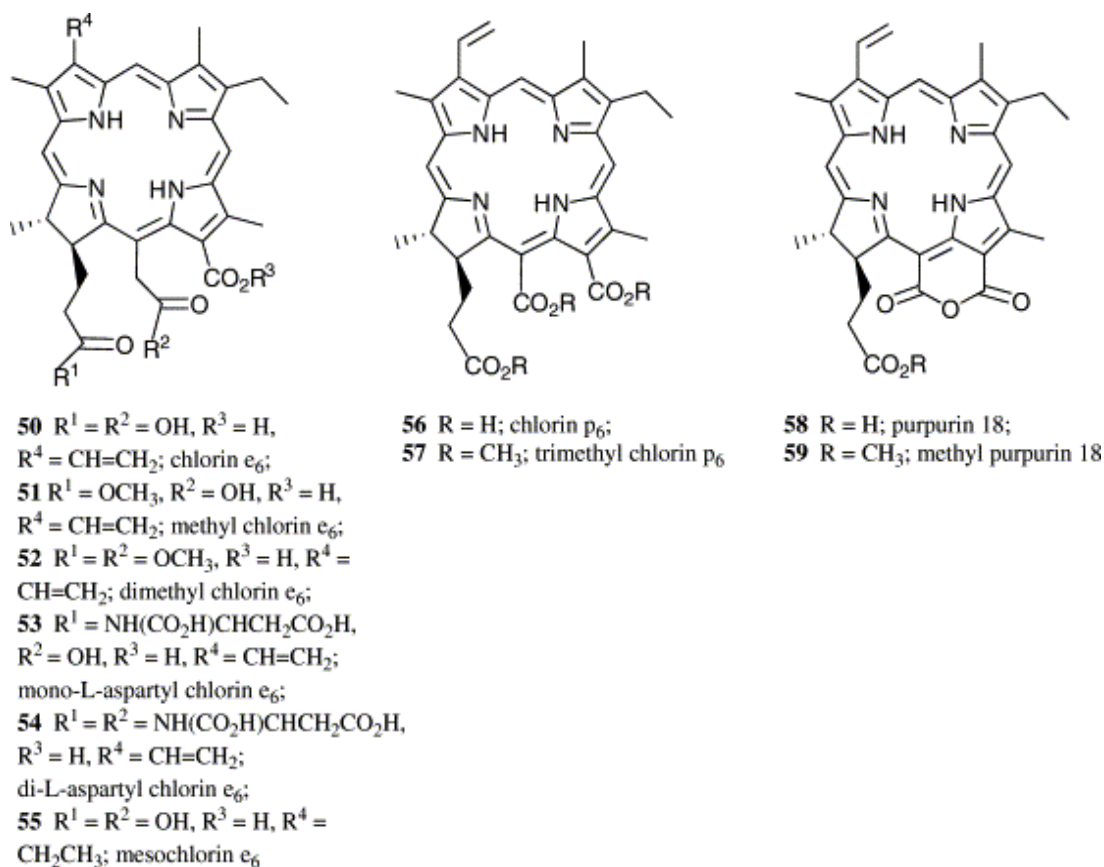


Figure 23. Completely synthetic chlorin and bacteriochlorin derivatives that have been proposed for PDT. (source; Nyman and Hynninen, 2004).

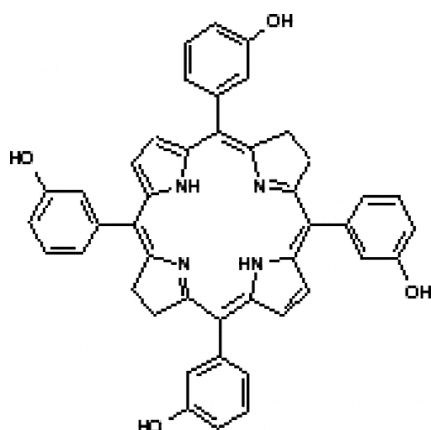


Figure 24. Chemical structure of meta-tetra-hydroxyphenyl bacteriochlorin (mTHPBC)

1.6.4.3 Other porphyrin- and chlorophyl-derived photosensitisers:

Porphines are synthetic porphyrins that have been shown to have high photosensitising and anti-mitotic potency in tumours (Kreimeier-Birnbaun, 1989). Topical TPPS4 (tetra-sodium-meso-tetraphenylporphine-sulfonate) has been evaluated intensively for the treatment of skin tumours (Santoro *et al*, 1990). The hydrophilic compound is activated by light at 630 nm and localizes preferentially in the tumour stroma (Kessel *et al*, 1987). It is 25-30 times more effective than HPD and Photofrin as photosensitiser (Calzavara-Pinton *et al*, 2006). The major limitation of TPPS4 is its neurotoxicity (Lui and Anderson, 1992).

Benzoporphyrin derivative monoacid ring A (BPD-MA), also known as Verteporphin (Visudyne®, Novartis AG, basel) is a semi-synthetic porphyrin derived from protoporphyrin (Henderson and Dougherty, 1992) (see **Figure 25**). This photosensitiser has poor solubility but can be formulated successfully either in liposomes or emulsions (Knabler *et al*, 1999). The absorbance peak for PDT occurs at 650 nm. BPD-MA has important applications in the treatment of choroidal neovascularisation in age-related macular degeneration (AMD). The photosensitiser BPD-MA has also found potential applications in dermatology especially for PDT of BCC and SCC (Kalka *et al*, 2000). Recent studies (Phase III clinical trials, see Gerber-Leszczynszyn *et al*, 2005) have shown that injectable BPD-MA possesses a significant and rapid accumulation in the tumour low residual skin photosensitivity and the overall cosmetic effect is acceptable.

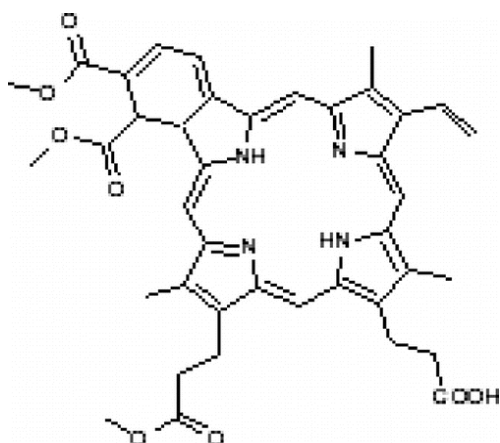


Figure 25. Chemical structure of benzoporphyrin derivative monoacid ring A (BPD-MA)

Porphycenes are structural isomers of porphyrins and texaphyrins are modified porphyrins in which a phenyl ring replaces one pyrrole ring. Both porphycenes and texaphyrins generate $^1\text{O}_2$ efficiently (Kreimer-Bimbaum, 1989). Porphycenes possess high fluorescence yields and show a 10-fold increase in light absorption at 630 nm when compared with HPD (Kreimer-Bimbaum, 1989). As a result they are considered potentially useful as diagnostic tools (Palumbo, 2007). The porphycene dye 9-acetoxy-2,7,12,17-tetrakis-methoxyethyl-porphycene has also been assayed for potential topical application in skin lesions (Karrer *et al*, 1997) (see **Figure 26**).

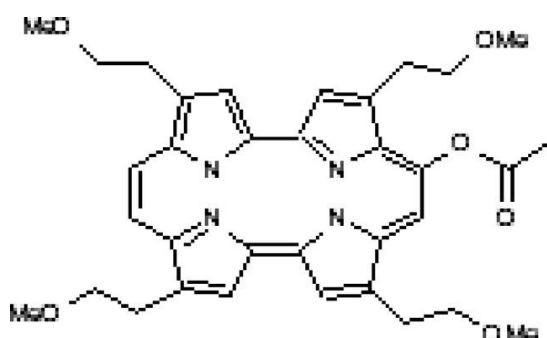


Figure 26. Chemical structure of 9-acetoxy-2,7,12,17-tetrakis-methoxyethyl-porphycene.

Texaphyrins can easily be complexed with large metal cations such as Lu(III) to give metal complexes that are photoactive *in vivo* (Nyman and Hynninen, 2003). The Lu(III)-complex of a texaphyrin (Lu-Tex, Lutryn®, Pharmacyclics Inc., CA, USA; see **Figure 27**) is a very stable hydrophilic photosensitiser. It is a highly fluorescent dye and absorbs strongly at 732 nm (Woodburn *et al*, 1996), and rapidly accumulates in neoplastic tissue allowing irradiation as early as 2-4h after drug administration (Kalka *et al*, 2000). The lack of significant persistent skin phototoxicity is another outstanding characteristic of these compounds (Sessler and Miller, 2000). Preliminary results based on the therapy of various malignancies, such as recurrent breast cancer, invasive SCC, malignant melanoma, Kaposi's sarcoma and BCC have demonstrated best response rates in breast cancer lesions and partial responses in other treated tumours (Renschler *et al*, 1997). Lu-Tex has also been subject of clinical trial in patients with locally recurrent prostate cancer (Palumbo, 2007). Nevertheless Lu-Tex can cause severe cutaneous pain during PDT (Kreimer-Bimbaum, 1989).

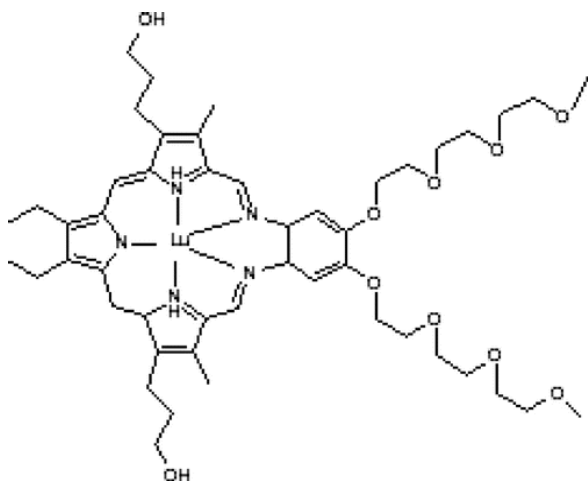


Figure 27. Chemical structure of Lu(III) texaphyrin (Lu-Tex).

Pheophorbides are a group of chlorophyll-derived photosensitisers. Within this group of photosensitisers Palladium-bacteriopheophorbide has been used to treat human prostatic small cell carcinoma (Koudinova *et al*, 2003) and 2-[1-hexyloxyethyl]-2-devinyl pyropheophorbide-a (HPPH) has been assessed successfully in Phase II clinical trial (NCT00281736) for the treatment of oesophageal cancers with very mild skin photosensitivity declining rapidly within days (Bellinier *et al*, 2006). HPPH-23 is another promising photosensitiser which has been successfully used in PDT of SCC in animal studies. It also appears to be a potent drug for the management of skin cancer. HPPH-23 can also be cleared rapidly from normal skin (Magne *et al*, 1997).

Finally, Hypericin (see **Figure 28**) a naturally occurring substance found in plants (*Hypericum perforatum*) is a promising PDT agent for the treatment of cancer with strong photosensitising properties, a high yield of $^1\text{O}_2$ and fluorescence emission. These properties has made hypericin a powerful diagnostic tool for example in the photodiagnosis of small tumor of urothelium (Kubin *et al*, 2005; Olivo *et al*, 2003; Palumbo, 2007).

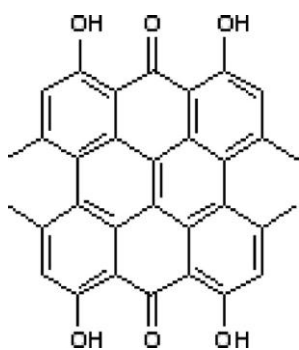


Figure 28. Chemical structure of Hypericin

1.6.5. Topical photosensitising mediators: α -Aminolaevulinic acid and esters

Another method of photosensitising cells is to stimulate the synthesis of endogenous photosensitisers notably the haem immediate precursor PPIX. This method of sensitising tumours with an intermediate of the haem biosynthetic pathway *i.e.* ALA was originally proposed by Malik and Lugaci in 1987.

PPIX is an efficient photosensitizer and its rate of formation is dependent on the rate of ALA synthesis (see **Figure 29**) from glycine and succinyl coenzyme A which in turn is regulated in a negative feedback manner by the concentration of the final product heme.

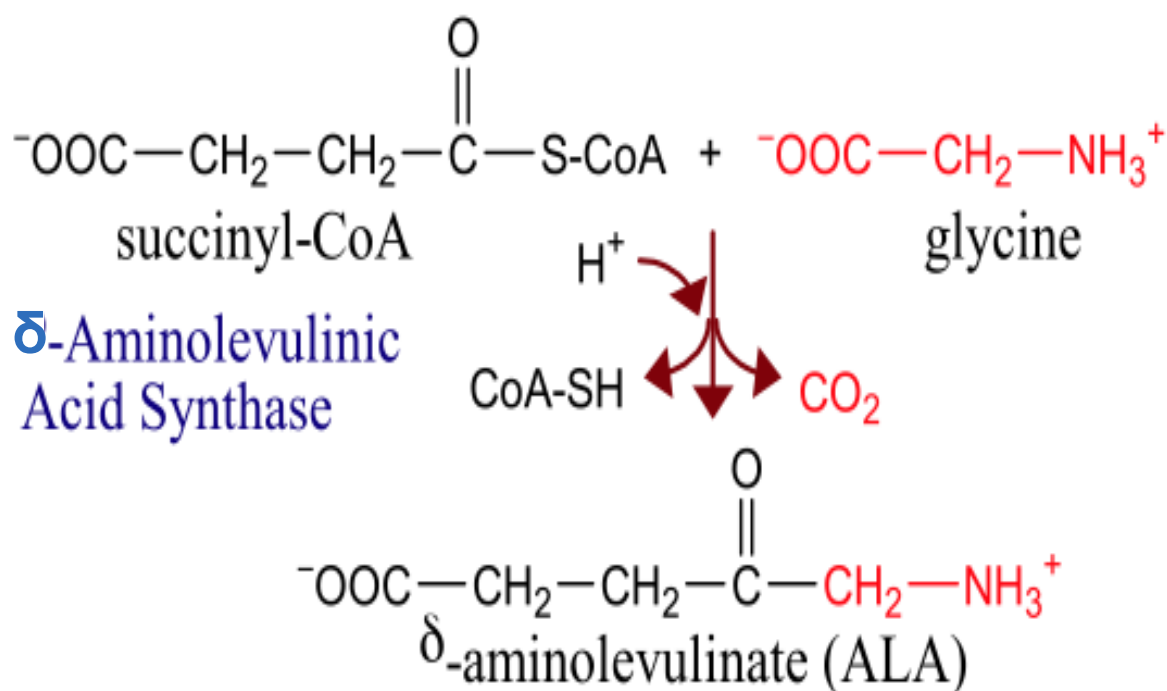


Figure 29. Synthesis of α -aminolaevulinic acid (ALA) from glycine and succinyl coA by ALA-synthase.

As the conversion of PPIX to haem is rather slow, administration of exogenous ALA can bypass the negative feedback control of haem biosynthesis, leading to intracellular accumulation of photosensitizing concentrations of PPIX. The resulting photosensitization provides a basis for using ALA-induced PPIX for photodynamic therapy (PDT) of cancer

(Kennedy *et al*, 1990; Wolf and Kerl, 1991; Peng *et al*, 1992). Typically, ALA supplementation into tumour tissue is followed by red (630-635 nm) or blue (380-420 nm) light treatment of the tumour tissue. In the ideal situation, photochemically generated ROS thus formed will destroy the tumour tissue while sparing the surrounding normal tissue.

The fluorescence of ALA-induced PPIX can also be used for the purpose of diagnostic detection of tumor cells, such as in early stages of skin malignancies. This procedure, termed photodynamic diagnosis (PDD), aids delineating poorly defined tumor borders before the use of invasive treatment modalities.

1.6.5.1. Haem biosynthesis pathway

Figure 30 illustrates the haem biosynthetic pathway. Briefly, the enzymatic condensation of glycine and succinyl-CoA to ALA by ALA-synthase (ALAS) is the initial and rate-determining step of haem synthesis, which is regulated by haem through a negative feedback mechanism. The enzyme ALAS is located on the matrix side of the inner mitochondrial membrane loosely associated with the membrane (May and Bawden, 1989). Once in the cytoplasm, ALA is metabolized by ALA dehydratase (ALA-D), which induces the condensation of two molecules of ALA to yield porphobilinogen (PBG). The combined action of PBG deaminase (PBGD) and uroporphyrinogen III co-synthase condenses in a head-to-tail manner four molecules of PBG and cyclises the tetrapyrrole chain to form uroporphyrinogen III (Xu *et al*, 1995). Uroporphyrinogen decarboxylase then removes four acetic acid carboxy groups to form coproporphyrinogen III (Moore *et al*, 1987). The subsequent steps in the mitochondrion result in the build up of PPIX. The final step of haem synthesis is the incorporation of iron into PPIX which is catalysed by ferrochelatase in the inner mitochondrial membrane. If an excess of PPIX is synthesized, it can diffuse from the

mitochondrion into the endoplasmic reticulum and also into the plasma membrane, both which are the other known sites of cellular damage through PDT (Barr *et al*, 2002).

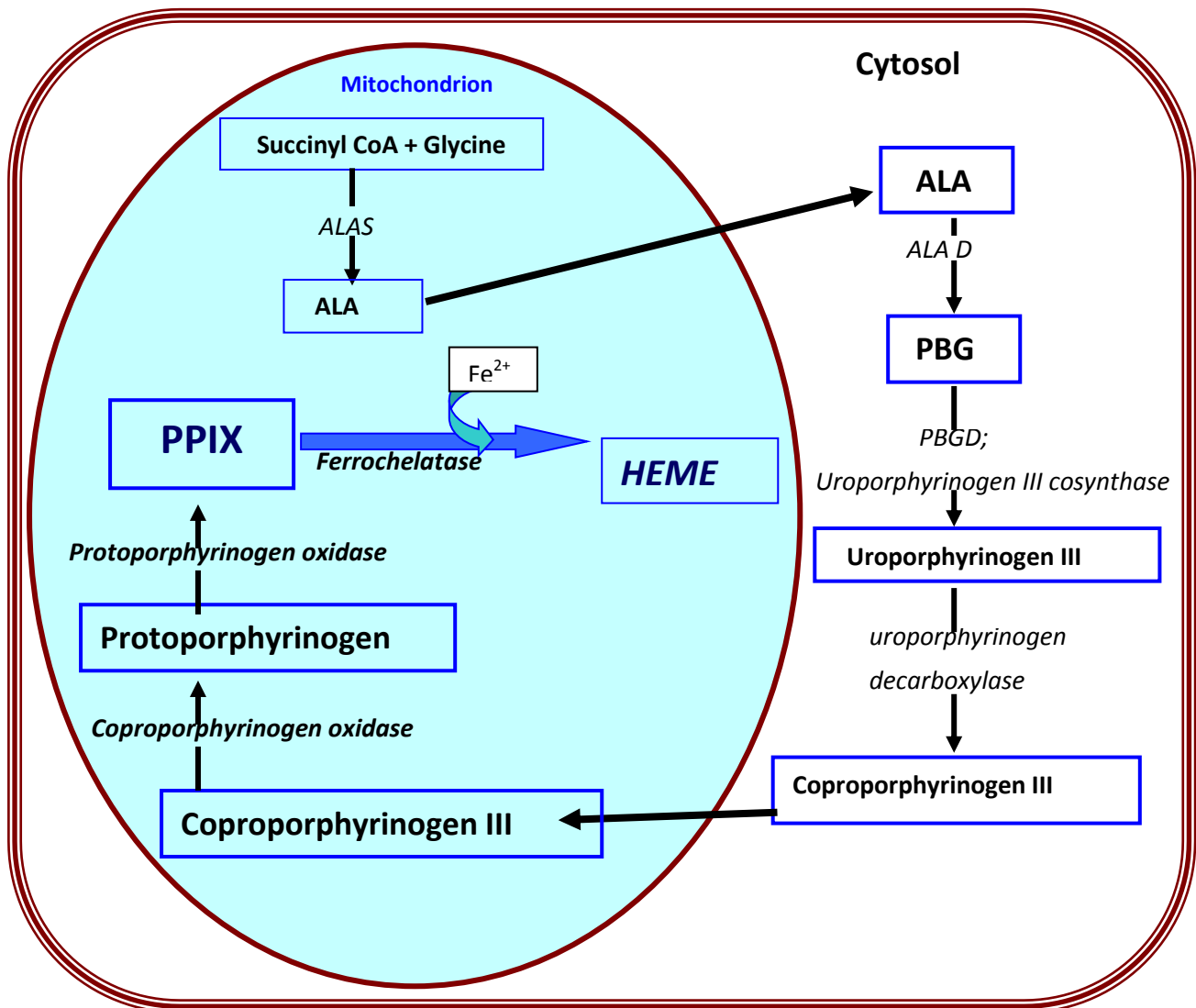


Figure 30. Haem biosynthetic Pathway (Modify haem and the image)

1.6.5.2. Advantages of topical ALA-PDT

The main advantage of using topical ALA-PDT is the absence of prolonged cutaneous photosensitivity when compared to systemic HPD/Photofrin-PDT (Peng *et al*, 2001). While with ALA-PDT there is no risk of skin photosensitivity after 24h, with HPD/Photofrin PDT, the photosensitivity of the skin is sustained for at least 4-6 weeks after treatment and unlike ALA-PDT modality, patients treated with HPD/Photofrin-PDT often stay in hospital for few days after the therapy. Furthermore ALA-PDT is a relatively low cost modality as ordinary lamps with suitable filters can be used whereas HPD/Photofrin-PDT treatment is rather expensive as a laser is used in most cases. ALA-PDT has also a high selectivity leaving the surrounding normal skin intact and functional. Furthermore several separate lesions can be treated simultaneously by ALA-PDT and if necessary the same lesions can be repeatedly treated. Cosmetic results of ALA-PDT is also superior to conventional modalities (Peng *et al*, 2001), however compared to HPD/Photofrin-PDT, it is more efficient for superficial lesions than nodular lesions (Peng *et al*, 1997). The optimal application time and the ALA concentration in ALA-PDT, depends on the characteristics of the targeted cells. The abnormal *stratum corneum* shows increased permeability to ALA, which is partly responsible for the tumor selectivity. Ironically, this phenomenon contributes to the major limitation of ALA-PDT; the poor ALA penetration through intact keratinised surfaced layers of nodular tumours could be the reason for the therapeutic refractivity of this malignancy with ALA-PDT.

1.6.5.3. ALA and ALA-esters uptake and cytotoxicity

A partial drawback of ALA as a photosensitiser is its hydrophilic nature, which limits its penetration into tissues. In mammalian cells ALA is taken up by active transport mechanisms, *i.e.* Na^+/Cl^- -dependent β -amino acid, including glycine and γ -aminobutyric acid (GABA) transporters (Rud *et al*, 2000). These systems require energy, depend on pH and temperature and although they are slightly accelerated in tumour cells, in general they are considered as saturable and slow processes (Klock and van Henegouven, 1996; Gaullier *et al*, 1997; Uehlinger *et al*, 2000). This problem has been in part reduced by the use of lipophilic ALA-esters notably methyl-aminolaevulinate (MAL) which accumulates selectively as ALA in neoplastic tissue and seems to penetrate the skin rapidly and deeply. For example it has been shown that, in WiDr adenocarcinoma cells, MAL is taken up by active mechanisms mainly through transporters of non-polar amino acids such as L-alanine, L-methionine, L-tryptophan and glycine (Gederaas *et al*, 2001). Most importantly, MAL is also taken up by passive transmembrane diffusion. This mechanism that does not require energy, is unsaturable and is also very efficient in normal cells and even more so in neoplastic cells. The plurality and efficiency of these diverse mechanisms determine the enhanced penetration characteristics of MAL in comparison to ALA. This difference is even more significant in malignant cells (Peng *et al*, 1997). Soon after cell penetration, MAL is rapidly demethylated to ALA and therefore the subsequent metabolic steps remain the same (Gaullier *et al*, 1997). In addition, ALA-ester derivatives achieve the maximum in intracellular PPIX concentration which leads to a shorter incubation time of 3 h compared with ALA (4-6 h) (Juzeniene *et al*, 2002).

1.6.5.4. Light Sources for topical ALA/MAL-PDT

Table I provides an overview of light sources used in PDT. The first light sources used in PDT were non-coherent light sources (Klein *et al*, 2008). Since then, numerous studies have demonstrated the effectiveness of long-pulsed dye lasers (Klein *et al*, 2008). Along with much shorter exposure times, the laser sources also allow the user to select the optimal wavelength. Flash-lamps are also commonly used in PDT of AK, photorejuvenation and *acne vulgaris* (Alexiades-Armenakas, 2006).

Table 1: Common light sources for PDT

Non-coherent lamps	Laser	Other
- Xenon lamps (400-1200 nm)	- Pulsed dye laser (PDL) (585 nm)	- Intense pulsed light (IPL) (500-1200 nm)
- Halogen lamps (560-800 nm)	- Long-pulsed dye laser (595 nm)	- Light emitting diode (LED) (631 ± 2 nm)
	- Argon dye laser (ADL) (450-530 nm)	-Fluorescence diagnosis systems
	- Diode laser (800-1000 nm)	

Note: Modified from Klein *et al*, 2008

Photodynamic management of dermatologic conditions is simplified by the accessibility of the skin to light application and leaves the option to use any light device with the appropriate spectrum corresponding to the absorption maximum of the photosensitiser. The popular filtered slide projectors are now gradually being replaced by non-coherent sources constructed especially for their use in PDT. The PDT 1200 lamp (Waldmann) is a metal halogen lamp which emits 600 to 800 nm radiation at light power density; keeping light

exposure times within practical limits (Stables, 1995). Another alternative light device is the short-arc xenon lamp, tunable over a band width between 400 and 1200 nm. (Whitehurst *et al*, 1993). Non-coherent light sources are safer, easy to use, and less expensive. They can produce spectra of wavelengths to accommodate various photosensitisers. The broad light beam produced by non-coherent lamps is useful for the treatment of large lesions using ALA-PDT. However the lower radiation power at the periphery of the treated lesion as opposed to the centre of the irradiation area may result in insufficient treatment of the tumour borders (Karrer *et al*, 1995). When multiple lesions are located on a convex surface (e.g. multiple AK on the scalp), it is necessary to deliver light from multiple directions to ensure the illumination of all the lesions.

Non-coherent light sources can be used in conjunction with optical filters to output selective wavelengths. The disadvantages of conventional lamps include significant thermal effect, low light intensity and difficulty in controlling light dose. However, nowadays, most of these drawbacks can be overcome by careful engineering design. For example it is known that non-laser light sources emit significant fluences of infrared radiation together with light useful for PDT (Stringer *et al*, 1995). Infrared radiation should be filtered out to avoid hyperthermia, although some investigators find that mild hyperthermia (40-42°C) acts additively or synergistically with PDT (Waldow and Dougherty, 1984; Kimel *et al*, 1992). To avoid hyperthermia, a fluence rate lower than 150 mW/cm² is recommended (Peng *et al*, 1997).

As it can be seen in **Figure 18**, PPIX has its largest absorption peak in the blue region of 405-410 nm (Soret band), with smaller absorption peaks at 505, 540, 580 and 630 nm. Most light sources for ALA/MAL-PDT aim to utilise the 630 nm in the red region to improve the tissue penetration. However a blue fluorescent lamp called the BLU-Ulight illuminator (DUSA Pharmaceuticals, Inc), (peak emission 417 nm) is now routinely used in Levulan ALA-PDT of AK in USA. There are now several reports that blue, green and red light can each be

effective in topical PDT of AK, but more deeply penetrating red light is superior when treating BD and BCC (Morton *et al*, 2002).

As ALA-PDT is mostly used in the treatment of tumours at the surfaces of organs (i.e. the skin, bladder and aerodigestive tract), lamps may be as well suited as lasers. In contrast to traditional incandescent lamps, lasers provide the exact selection of wavelengths matching the absorption of the photosensitiser and the precise application of light. The tunable argon-dye laser (ADL) is composed of an argon laser that emits a blue-green beam in the 450-530 nm range, pumping a dye laser to obtain light in the range of desired wavelength (Kalka *et al*, 2000). The ADL system emits light continuously and is tunable to suit the absorption properties of various photosensitising compounds (350-700 nm), representing the most popular light device used in PDT (Fisher *et al*, 1995).

Non-laser sources are popular in topical PDT, possessing the advantages over lasers of being inexpensive, stable, and easy to operate, requiring little maintenance and providing wide area illumination fields. Retrospective comparison of laser and filtered broadband sources suggests equivalent efficacy in topical PDT (Clark *et al*, 2003). Currently a range of non laser light sources, doses and irradiations continues to be used in ALA-PDT, in comparison to MAL-PDT which typically involves light emitting diode (LED) source.

In the last few years, LED sources have undergone considerable development, with improvements in design making these relatively inexpensive sources convenient for wide area irradiation and popular for patient use, *e.g.* the Aktilite 16 and 128 (Galderma, France) and the Omnilux (Phototherapeutics Ltd, Altincham, UK). These LED sources match the 630/635 nm activation peak of PPIX while excluding the extraneous wavelengths in broadband sources, thus permitting shorter irradiation times. Biophysical calculations indicate that LED source with peak emission of 631 ± 2 nm may have a deeper PDT action in

tissue than a filtered halogen type lamp of 560-740 nm emission, and hence LED may be more effective in treating the deeper parts of tumours (Juzeniene *et al*, 2004). LED can generate high energy light of desired wavelengths and can be assembled in a range of geometries and sizes. Babilas *et al* (2006) compared the effectiveness of a non-coherent light source with an LED system. *In vitro* cultured human epidermal keratinocytes were incubated for 24h with various ALA concentrations and then exposed to either incoherent or LED light. No significant difference was observed in cytotoxicity induced by the two light sources. In a subsequent *in vivo* investigation, patients with at least two AK lesions were exposed to these light sources (i.e. half to each light source). There was again no significant difference in remission rates, pain or cosmetic results (Klein *et al*, 2008). In another study, Babilas *et al* (2007) compared an LED system with a flash-lamp (VPL, variable pulsed light; Energist Ultra VPL™, Energist Ltd, Swansea, UK) for the MAL-PDT of AK. Both lamps were similarly effective, although pain during and after treatment was significantly lower on the side of face treated with the flash-lamp than on the side exposed to LED.

A series of studies have suggested that pulsed light therapy may be useful for treatment / adjunctive treatment in topical PDT of *acne vulgaris*, AK and photorejuvenation (Morton *et al*, 2008). However a recent controlled investigative study performed in healthy human skin *in vivo* following microdermabrasion and acetone scrub, showed that two pulsed light sources previously reported in PDT, the PDL and a broadband flashlamp filtered intense pulsed light (IPL), produced evidence of minimal activation of photosensitiser, with a dramatically smaller photodynamic action than seen with a conventional wave broadband source (Strasswinner and Grande, 2006). Furthermore IPL and PDL sources deliver intense light in periods (i.e. 20 ms), which might suppress oxygen consumption (Kawauchi *et al*, 2004).

Buchczyk *et al* (2001) also compared the effectiveness of ALA-PDT in a human cultured skin fibroblasts with three different light sources of (i) red light source (570-700 nm;

PDT1200 illuminator, Waldmann Lichttechnik, Germany) at an intensity of 50 mW/cm²; (ii) green light source (545 ± 3 nm; PDT Green Light, Saalman, Herford, Germany) at intensities of 10-20 mW/cm²; (iii) UVA light (320-400 nm; UVA700 illuminator, Waldmann Lichttechnik, Villingen, Germany) at intensities of 44 (without ALA) and 10 mW/cm² (with ALA). The results showed that compared with red light source, UVA-ALA-PDT was 40-fold more potent in killing cultured human skin fibroblasts and still 10-fold more potent than ALA-PDT with green light. The high cytotoxicity of UVA-ALA-PDT relied on the efficient formation of ¹O₂ as was demonstrated with modulators of ¹O₂-half-life. So, ALA-PDT in the UVA region might be preferable to red or green light ALA-PDT for treatment of readily accessible tissue because of its higher efficiency. The latter study further justifies the use of blue light for ALA-PDT of superficial skin lesions such as AK. Although in most cases of ALA-PDT, light at 630 nm is applied, light in the Soret band (410 nm) is a powerful alternative as it would give the largest cell inactivation down to about 2 mm from the surface in human skin and muscle tissues as well as in superficial BCC. However at depths exceeding 2 mm, 635 nm light might be a better option than blue light (Szeimies *et al*, 1995; Peng *et al*, 1997).

1.6.5.5. Dermatologic applications of ALA/MAL-PDT

Non-melanoma skin cancer (NMSC) is the most common cancer in UK, with official figures reporting over 72,400 cases diagnosed only in 2004 (Neville *et al*, 2007). The vast majority of NMSC is Basal Cell Carcinoma (BCC) which comprises 75% of all NMSC cases (Neville *et al*, 2007). Squamous Cell Carcinoma (SCC) is the second most common type of skin cancer in this country (i.e. 20% of skin cancers) (Neville *et al*, 2007). In the USA, one million new cases of NMSC are diagnosed each year and of these 80% are BCC and 16% are SCC

(Neville *et al*, 2007). The incidence of SCC is increasing across Europe, the USA and the Southern hemisphere (Neville *et al*, 2007; Schmook and Stockfleth, 2003) and the incidence of BCC continues to rise in Australia; it is estimated that NMSC affects at least 1-2% of the population annually (Schmook and Stockfleth, 2003; Marks, 1997).

BCC may be categorized into three major growth patterns: nodular (nBCC), superficial (sBCC) and morphoeiform (or sclerotic; mBCC). Superimposed on any of these growth patterns may be ulceration or pigmentation. Metastasis is extremely rare and the morbidity associated with BCC is related to local tissue invasion and destruction, particularly on the head and neck. Most sBCCs will progressively enlarge over months to years and with time, may adopt nodular or even sclerotic growth patterns. nBCCs that are often found on the head and neck of old subjects will also progressively enlarge with tendency to deep extension and ulceration. In contrast, mBCCs have a sclerotic growth pattern with the appearance of pale scar that tends to be deeply invasive, making surgical and non-surgical treatments less effective (Albert and Weinstock, 2003).

Exposure to UV is the most important risk factor of NMSC and is related to sun-exposure habits. Light complexion, increasing age, male gender, immunosuppression, ionizing radiation and psoralen phototherapy and precancerous skin lesions are other host factors (Albert and Weinstock, 2003). Among precancerous skin lesions, Aktinic keratoses (AK) that are potential precursors of SCC are very common with an incidence about 10-fold that of BCC (Harvey *et al*, 1996). In Australia, it has been estimated that 60% of the population over the age of 40 years will have at least one AK lesion (Schwartz, 1997). AK lesions could affect the face, scalp, hands and forearms in the form of small, dry, rough yellow-brown lesions with well-defined scales that do not flake off. They may become thick and horny and sometimes bleed. Pathological evidence suggests that AK should be regarded as very early SCC (Czarnecki *et al*, 2002). As 3-4% of SCCs metastasize (Lober and Lober, 2000), it has

been suggested that AK should be treated early to avoid malignancy and the need for more extensive treatment.

Among NMSC conditions, Bowen's disease (BD) is also a persistent form of intra-epidermal (*in situ*) SCC, which appears as an enlarging, well-demarcated, erythematous plaque with an irregular border, a crusted or scaling surface and potential for extending along the cutaneous appendages. Nevertheless BD has a small potential for invasive malignancy (Morton, 2005).

As the incidence of NMSC increases, so does the number of modalities used to treat this condition. Surgery is the most common approach with the Mohs micrographic surgery being the best treatment so far, but owing to the time and expense involved with this procedure, it is indicated only in patients with aggressive tumours. Apart from surgical excision, cryotherapy, curettage and electrocautery are practiced. Radiation therapy is also effective but its use is limited because of the side effects. Several regimens of chemotherapy have been clinically applied for the treatment of NMSC but have turned out to be insufficient at improving the prognosis (Cassileth and Chapman, 1996; McCann 1997). Further non invasive options for NMSC include topical chemotherapeutics, biological immune response modifiers and retinoids which can be used particularly in patients with superficial tumors. Treatments so far are tailored to tumor type, location, size and histological pattern but with increasing incidence of NMSC, there is a clear need to design new non invasive treatments to target cancer cells more generally at all stages of tumorigenesis.

PDT with topical ALA/MAL formulations represents one of the most promising treatments for NMSC (Cairnduff *et al*, 1994 ; Fink-Punches *et al*, 1997; Omrod and Jarvis, 2000;. Peng *et al*, 1997; Lehmann, 2007; Morton *et al*, 2001; Morton, 2005).

MAL (Metvix®, Photocure ASA, Oslo Norway and Galderma SA. Paris, France) is presently approved for use in Europe with combination with red light for treating AK, superficial and nodular BCC and BD. In the United States, a combination of an alcohol-containing ALA solution in a special applicator (Levulan Kerastick®, DUSA Pharmaceuticals Inc., USA) with blue light (417 nm) has been approved for clinical treatment of AK (Klein et al, 2008). Clinical studies have revealed that overall clearance rates for the treatment of AK with ALA/MAL-PDT ranges from 50% to 71% when one treatment is performed and up to 90% when two or more treatments are performed. Clearance rates for BCC using ALA/MAL-PDT range from 76-97% for superficial tumors to 64-92% for nodular BCC (Tierney *et al*, 2009).

Because of the significant results obtained from preclinical and clinical studies (Hongcharu *et al*, 2000), in 2003, ALA-PDT was also approved for moderate inflammatory *acne vulgaris* in USA.

Preclinical and clinical studies have also demonstrated the great potential of ALA/MAL-PDT for treatment of Mycosis fungoides (Stables *et al*, 1997). Other dermatological applications include human papilloma virus-associated cutaneous pathologies, lymphocytoma cutis, leishmaniasis, alopecia areata, erythroplasia of Queyret and benign familial pemphigus and hidradentis suppurativa (see Tierney *et al*, 2009; Kalka *et al*, 2000; Huang 2005). A number of other non-malignant conditions such as psoriasis, viral warts and hair removal have also been under clinical investigation internationally for more than a decade (Robinson *et al*, 1999; Stender *et al*, 1999; Stender *et al*, 2000; Hongcharu *et al*, 2000; Robinson *et al*, 1999; Huang, 2005). Clinical investigations of ALA-PDT have also extended to basaloid follicular hamartomas, cutaneous T-cell lymphoma and sebaceous gland hyperplasia in recent years (Leman *et al*, 2002; Horio *et al*, 2003; Coors *et al*, 2004; Gold *et al*, 2004; Oseroff *et al*, 2005). In addition to preventing the development of scars and dyspigmentation that occurs with surgery, ALA-PDT has the added benefit of photorejuvenation (Alster *et al*, 2005) and

has been used as a treatment to soften (Alster *et al*, 2005) the appearance of acne scars as well as fine lines and wrinkles of photodamaged skin (e.g. Alster *et al*, 2005; Dover *et al*, 2005).

1.6.5.6. Approaches to improve the uptake of ALA and MAL from skin lesions for efficient PDT response

Table 2 provides the product name and ingredients of some ALA preparations used in PDT of skin lesions. It is clear that optimisation of topical formulations of ALA and MAL will almost certainly improve the efficiency of ALA/MAL-mediated PDT. Christiansen and colleagues (2007) recently published a study on the fluorescence pattern of 20% ALA in a cream vehicle when compared to 0.5% and 1% ALA in a liposomal vehicle using ten young subjects with healthy skin. The comparison of fluorescence values between the two preparations showed that the maximum fluorescence for liposomal cream was already achieved after 2h, however in cream-based ALA, the maximum effect was only reached after 8h. The superior penetration of liposomal formulations of ALA may also lead to significantly decreased ALA concentration needed for PDT. Several PhaseII / III clinical trials are now underway for the use of liposomal or nanocolloidal ALA (*e.g.* BF-200 ALA, Biofrontera AG, Leverkusen, Germany) preparations for the treatment of AK (Klein *et al*, 2008).

Hyperkeratosis is often the reason for a poor response to topical ALA/MAL-PDT of skin lesions notably AK and BCC. Limited uptake of topically applied ALA/MAL and suboptimal production of PPIX may account for these differences. A time-consuming aspect of ALA-PDT of skin lesions is the removal of crusts with a curette (keratolysis) to improve penetration and decrease the incubation time of ALA or MAL (Thissen *et al*, 2000; Morton, 2003; Moore and Allan, 2003). Overnight incubation with an ointment for easy mechanical

removal might also be beneficial. Other modalities include the use of keratolytics, tape stripping, microdermabrasion or laser ablation (Klein *et al*, 2008) Penetration enhancers may also be beneficial as they may alter the composition or organisation of the intracellular lipids of the *stratum corneum*. Several studies have been performed on the use of DMSO, azone, glycolic acid, oleic acid and iontophoresis to increase the penetration of ALA (Ziolkowski *et al*, 2004; Orenstein *et al*, 1996; Ibboston *et al*, 2006; Lopez *et al*, 2003). One possibility is to administer ALA via topically applied patch (PD P 506, photonamic GmbH & Co KG, Wedel Germany). This possibility is presently subject of a multicentric Phase II clinical trial in Germany with patients suffering with AK (Klein *et al*, 2008; Donnelly *et al*, 2010).

Table 1: ALA Preparations

Product Name	Ingredients
Metvix®	16% methyl-5-amino-4-oxopentanoate as hydrochloride
Levulan Kerastick® (not approved in Europe)	20% aminolevulinic acid hydrochloride
Magistral preparation	20% ALA gel/cream/emulsion
PD P 506 A (not yet approved for clinical use)	5-ALA released from Bandage
BF-200 ALA (not yet approved for clinical use)	5-ALA nanoemulsion

Note: Reproduced from Klein *et al*, 2008

1.6.5.7. Factors influencing the efficiency of ALA-PDT

Hua *et al* (1995) have investigated the mechanism(s) of ALA-based PDT in order to evaluate its relative effectiveness in controlling tumour growth *in vivo*. They have demonstrated that PPIX is the predominant porphyrin species formed in tumors after ALA administration. Thus ALA-PDT efficacy depends to a great degree on the rate of PPIX synthesis. Unfortunately, the great variability in PPIX biosynthesis poses limitations to this therapy. The response rates of ALA-PDT vary widely due to suboptimal production of PPIX, which limits the overall anticancer photochemical reactions and accounts for the differences in clinical responses (Gerritsen *et al*, 2009; Ickowicz *et al*, 2004). It appears that the differential expression levels of the rate-limiting enzymes in heme biosynthesis pathway between non-malignant and malignant cells, is the main factor affecting the accumulation of PPIX in those cells (Greenbaum *et al*, 2002). These key enzymes are ALAS, ALAD, PBGD and ferrochelatase. ALA supply circumvents the first enzyme ALAS, thus the synthesis rate is largely dependent on the other enzymes especially PBGD, ALAD and ferrochelatase as demonstrated in a series studies in the field (*e.g.* Grinblat *et al*, 2006; Ickowicz *et al*, 2004; Miyake *et al*, 2009; Feuerstein *et al*, 2009).

The accumulation of PPIX in the cells is not only influenced by the ratio of ALA conversion to PPIX but is also related to the ferrochelatase-mediated insertion of ferrous iron into the porphyrin macrocycle to produce the heme molecule. It has been shown that differences in iron availability in cells may influence the patterns of PPIX accumulation *in vivo* (Licznarski *et al*, 1993). The selective accumulation of PPIX in some malignant cells is also attributed to their low ferrochelatase activity (Schoenfeld *et al*, 1988; El-Sharabasy *et al*, 1992). A correlation between cell proliferation rates and PPIX synthesis has also been suggested (Rebeiz *et al*, 1992; Malik *et al*, 1989), but these studies lack an explicitly stated proliferation rate. A clear model does not emerge from these studies, probably because of the difficulty in

comparing the results obtained from tumors of diverse origin, where the rate of PPIX accumulation is dictated by a varying combination of specific cellular and tissue characteristics. For the clinicians, standardized PDT protocols are needed; nevertheless, such protocols must be finely tuned for specific lesions depending on PPIX synthesis capacity in order to improve the clinical outcome. Therefore there is a strong requirement to find cell and tissue markers which will indicate which tumors are suitable for ALA/PDT, since the effectiveness of ALA in inducing PPIX in the cells is cell-, tissue- and organ-specific (Kennedy *et al*, 1990; Feuerstein *et al*, 2009). In 1995, Oseroff and coworkers (Rittenhouse-Diakun *et al*, 1995) used the transferrin receptors (TfRs, also designated CD71) of the cells as a marker for the intracellular level of iron in ALA/PDT. They showed that some activated normal and malignant lymphocytes that have increased CD71 expression, can be identified as cells highly susceptible to ALA/PDT and therefore could serve as a PDT target. However this marker may have limited usefulness because in some malignant cell lines, the presence of elevated TfR expression is unrelated to internal iron stores (Neckers, 1991; Reelfs *et al*, 2010).

Direct measurement of intracellular levels of LI in tumour cells is extremely difficult. Pourzand and coworkers (1999b) have demonstrated that the sensitive estimation of intracellular level of LI as monitored by the level of activation of cytosolic IRP-1 could serve as potential sensitive marker for developing PDT protocols since it identifies cells and tissues with a propensity to accumulate PPIX and it is therefore likely to predict the effectiveness of such therapies (Pourzand *et al*, 1999b).

Because of the clear role of iron in determining the efficiency of PPIX accumulation after ALA administration, iron chelator treatment combined with ALA-PDT has been proposed as an efficient mean to improve this therapy by decreasing the amount of available iron in the cells. Preclinical studies have already shown that iron chelators such as EDTA,

Desferrioxamine mesylate (Desferal®, Novartis, Basel, Switzerland), 2-allyl-2-isopropylacetamide and 1,10-phenanthroline have potential for increasing PPIX accumulation from ALA (Licznarski *et al*, 1993; Hanania and Malik, 1992; Iinuma *et al*, 1994, He *et al*, 1993; Berg *et al*, 1996; Liu *et al*, 2004). However these chelators are highly hydrophilic and therefore are not suitable for topical application. Studies from MacRobert and Curnow laboratories have introduced CP94 (1,2-diethyl-3-hydroxypyridin-4-one hydrochloride) that appears to be a more suitable iron chelating agent for dermatological ALA-PDT. CP94 possesses not only high specificity for iron but also a lower molecular weight and higher lipophilicity than DFO and EDTA (Smith *et al*, 1997; Liu and Hider, 2002). The effectiveness of this chelator in enhancing the PPIX levels following ALA administration has been shown in *in vitro* cell culture studies as well as in *in vivo* animal models and skin explants (Bech *et al*, 1997; Casas *et al*, 1999; Chang *et al*, 1997; Curnow *et al*, 1998, Curnow *et al*, 2006; Pye and Curnow, 2007; Blake and Curnow, 2010). The use of lipophilic strong iron chelators such as CP94 in combination with ALA/MAL may circumvent the problems of unresponsiveness of deep dermatological lesions in topical ALA-PDT and should also improve the efficiency of ALA-PDT in skin tumours with low response to ALA-PDT. In a recent study, Pye *et al* (2008) demonstrated the improved efficiency of MAL-PDT of nodular BCC when combined with CP94. (Campbell *et al*, 2004)

Although little information exists about the tissue penetration of ALA after topical application, the fluorescence of ALA-mediated PDT in normal and diseased human skin has been found to increase with time after topical ALA application, with a plateau of approximately 4-14 h, depending on ALA concentrations (2-40%) in formulations, amount of administration (30-50 mg/cm²), and application time (up to 24 h) used (Orenstein *et al*, 1995; Morton *et al*, 1995; Peng *et al*, 1997). In general, topical application of ALA alone for less than 4 h produces PPIX only at the site of ALA application, whereas the administration for a

longer period (up to 14 h) or combined with skin penetration enhancers (*e.g.* dimethylsulfoxide; DMSO) leads to a generalized photosensitization of the skin (Orenstein *et al*, 1995; Peng *et al*, 1997). Generally, ALA-induced PPIX fluorescence can not be detected in the skin 24 h after completion of topical ALA application. ALA itself does not seem to be toxic to tissues when concentrations <50% in water/oil emulsion by weight are topically applied for at least 48 h. Moreover, no evidence shows toxicity of ALA-mediated PPIX accumulation on tissues before light exposure (Peng *et al*, 1997). Cellular studies have demonstrated that ALA can be administered at very high concentrations without producing cytotoxic effects in the dark. At concentrations lower than 3 mM, no dark cytotoxic effects could be observed (*e.g.* Pourzand *et al*, 1999b; Berlanda *et al*, 2010). The intracellular production of PPIX is almost linear over the investigated incubation time (30h). PPIX formed from ALA is rapidly degraded, thus minimising the period of light sensitivity post PDT. Another advantage of ALA-induced PPIX is that it is only produced in living cells, which is beneficial for treatment of cells without production of ROS in surrounding extracellular matrix (Berlanda *et al*, 2010).

1.6.5.8. Side effects of ALA- and MAL-PDT

Topical ALA- or MAL-based PDT has considerably advanced the management of NMSC, providing a treatment option for AK, BCC and BD, with good clinical outcomes, low recurrence rates and enhanced cosmetic acceptability (Morton *et al*, 2002). However the most common and troublesome acute adverse event of topical PDT remains the burning or stinging pain that occurs during light exposure, and may continue post-exposure in a minority (Morton *et al*, 2002) Pain is restricted to the illuminated area and may reflect nerve stimulation and/or tissue damage by ROS, possibly aggravated by hyperthermia (Morton *et al*, 2002). Treatment

of psoriasis and viral warts in particular is frequently limited by pain (Schleyer *et al*, 2006). Pain appears more intense in large area lesions, with AK, BD and BCC covering an area of > 130 mm² significantly more painful to treat (Grapengiesser *et al*, 2002, Sandberg *et al*, 2006). In particular, patients receiving PDT of AK in the face and scalp often experience severe pain during treatment (Grapengiesser *et al*, 2002; Sandberg *et al*, 2006). There is therefore an imminent risk that patients are unable to complete the treatment session or will refrain from treatment in the future. Untreated lesions may increase the risk of developing SCC in the long run (Anwar *et al*, 2004; Salsche 2000). As topical ALA/MAL-based PDT has good cure rates in the treatment of extensive AK and gives excellent cosmetic results, it is necessary to find new strategies to reduce the pain experience to an acceptable level.

MAL may offer advantages over ALA in terms of its deeper skin penetration (up to 2 mm in depth) due to potentially enhanced lipophilicity and greater specificity for neoplastic cells (Peng *et al*, 2001). As ALA but not MAL is transported by γ -aminobutyric acid carriers, it has been speculated that MAL might provoke less nerve fibre stimulation and subsequent pain (Rud *et al*, 2000). The comparative PDT studies with topical ALA and its methyl ester derivative MAL are still ongoing. While some studies have demonstrated that pain was significantly higher in the ALA- than MAL-treated sites (both during and immediately after PDT), other studies have indicated that pain was greater in MAL-treated sites with more intense PPIX fluorescence and also with higher fluence rates with 20% of patients unable to complete the treatment due to unbearable pain (Wiegell *et al*, 2003, 2006 and 2008; Kasche *et al*, 2006; Kuipers *et al*, 2006; Moloney *et al*, 2007). More comparative studies are necessary to elucidate the pain associated with either compounds, as the high inter-subject variability within small subject group studies potentially obscure any existing relationship.

The degree of pain has also been related to intensity of light delivery; fractionated light doses increase tolerance of the procedure and may at the same time increase cure rates (Ericson *et al*, 2004; Babilas *et al*, 2007).

A range of techniques has been used in an attempt to reduce topical ALA/MAL-PDT-related pain, including local anesthesia and cooling the skin with fans or sprayed water (Halldin *et al*, 2009). Cold-water spray is used as pain relief routinely but is deficient when treating large areas such as the forehead and scalp. The difficulty in finding a pain relieving strategy has been shown in earlier studies, *e.g.* three studies have shown the inefficacy of topical anesthesia such as tetracaine gel, a mixture of lignocaine 2.5% and prilocaine 2.5%, or morphine gel 0.3% (Holmes *et al*, 2004; Langan and Colins, 2006; Skiveren *et al*, 2006). Blowing cold air (-35°C) on the treatment area during PDT also seems to fail to reduce the pain significantly (Pagliaro *et al*, 2004). Transcutaneous electrical nerve stimulation has been tried as pain reducing method but had limited effect. Subcutaneous infiltration anesthesia has been used during PDT of extensive AK of the cheek. The pain decreased but this type of anesthesia is not possible to use on the forehead and scalp. Applying local anesthesia to such areas is also inappropriate due to large number of painful injections needed. Nerve blocks have also been used to provide effective pain relief during PDT for extensive AK in the face (Paoli *et al*, 2008). A recent study has combined the benefits of supraorbital and supraocular nerve blocks of the latter study with nerve blocks of the greater and lesser occipital nerves to anesthetize both the forehead and scalp in order to alleviate pain during PDT in these areas. However in both studies intervariability was observed between the patients, emphasizing the need for individual considerations to find the most adequate pain relieving method for each patient.

1.7. Aims and objectives of the study

The present proposal responds to the urgent need of finding alternative efficient and more tolerable methods for topical ALA-based PDT of skin lesions such as AK. While the topical ALA- and MAL-based PDT of AK with prolonged visible or red light treatment provides efficient result and cosmetic outcome, discomfort from PDT is still a considerable hindrance with up to 20% of patients reporting pain as being ‘severe’ and ‘intolerable’. This often results on patients’ reluctance to this therapy, putting them at risk of developing SCC. Further optimization of the treatment protocol including light source, dose and duration therefore seems crucial to try and alleviate pain. In the present project, we hypothesized that the damaging effects of rapid release of labile iron by short pulses of low UVA doses following ALA-treatment may be exploited to provide a rapid mean to improve the effectiveness of ALA-PDT of skin lesions while reducing considerably the therapy time and the discomfort/pain associated with other light treatments such as prolonged high intensity visible light treatment. We therefore evaluated the response of spontaneously immortalized HaCaT keratinocytes to either single or two short pulses of UVA with 1h interval by evaluating the level of cell survival with the MTT viability assay and the mode of cell death with the dual Annexin V/propidium iodide flow cytometric assay. To ascertain that the cytotoxic effects observed were iron-dependent, we also evaluated the response of HaCaT cells that were iron-loaded prior to ALA-UVA split dose treatment. Our ultimate goal was to demonstrate that applying split low doses of UVA radiation in combination with ALA may be an effective intervention strategy to decrease considerably the time of light treatment and therefore reduce the pain associated with prolonged conventional topical ALA-PDT of skin lesions such as AK.

CHAPTER TWO

MATERIALS AND METHODS

2.1. Chemicals

All the reagents were purchased from Sigma-Aldrich Chemical Co. (Poole, UK) unless otherwise specified. All the cell culture materials were obtained from Life Sciences Technologies (Paisley, UK), except the fetal calf serum (FCS), which was obtained from PAA (Austria), Dulbecco's Minimum Essential Medium (DMEM) that was supplied by Gibco BRL Invitrogen Corporation (Life Technologies, UK) and Annexin-V-FITC that was purchased from Roche (UK). MilliQ water used to prepare phosphate buffered saline (PBS) and other stock solutions were issued from a Millipore purification system (MilliQ cartridge: Millipore, Bedford, MA) in order to minimize the presence of trace elements such as transition metals.

2.2. Cell Culture

2.2.1. Cell model

The cell model used in this study was the spontaneously immortalized human skin keratinocyte cell line HaCaT that was originally derived from an adult back in Boukamp's laboratory (Boukamp *et al*, 1988). This cell line maintains full epidermal differentiation capacity but remains non-tumorigenic (Boukamp *et al*, 1988). Cell culture procedures were conducted in a flow hood, (M.D.H, InterMed) using aseptic technique.

2.2.2. Preparation of media

The medium employed was 10% FCS-DMEM (high glucose Dulbecco's modified Eagle's medium) containing 0.25% sodium bicarbonate, 2 mM L-glutamine, and 50 IU/ml of each of penicillin / streptomycin (P/S). The FCS stock was heat-inactivated at 56°C for 45 min before use.

2.2.3. Trypsinisation

Cells were trypsinised once or twice a week and seeded back into T125 cm² flasks, so they were always in the exponential phase and at the required confluency of 70-80%.

For trypsinisation, the medium was removed and retained in a separate tube. The adherent cells were then washed with PBS buffer and incubated with 2 ml of 0.25% trypsin at 37°C under 5% CO₂, until detached (usually 5-10 min). Retained media was then added back to the flask in order to inactivate the trypsin. Next, the cells were centrifuged for 5 min at 1200 rpm in the Jouan B3.11 centrifuge. After removing the supernatant the cell pellet was resuspended in fresh 10% FCS-DMEM medium and cells were counted under a light microscope *i.e.* A small aliquot of the cell suspension was mixed with Trypan blue dye and then counted under microscope using a Neubauer-improved haemocytometer (Marienfeld, Germany). The cells were then diluted with fresh medium at the required density and incubated at 37°C under 5% CO₂.

2.2.4. Experimental set up

For experiments, 8.4×10^4 cells were seeded in 3 cm plates and grown for 3 nights at 37°C incubator under 5% CO₂ to reach 80% confluency. For accuracy, three 3 cm plates were used for each experimental condition.

2.3 Treatments

2.3.1. UVA irradiation

2.3.1.1. UVA lamp

The broad spectrum 4 kW lamp (Sellas, Germany) emits primarily UVA irradiation (significant emission in the range of 350-400 nm) and some near visible radiation longer than 400 nm. **Figure 2.1** illustrates the spectrum of the lamp. This lamp is suitable for ALA-PDT studies, as the absorption spectrum of PPIX is known to have a peak around 405-410 nm. Excitation at this wavelength produces an intense red emission with its maximum at 635 nm (Ericson *et al*, 2003). Furthermore blue light has been shown to be more potent than red light in activating PPIX (Peng *et al*, 1997).

The preliminary data from this laboratory have previously indicated that a 2h treatment with ALA concentration of 0.5 mM could significantly sensitise the HaCaT keratinocytes to the damaging effects of UVA (unpublished data, Pourzand's laboratory, personal communication).

2.3.1.2. UVA doses

The UVA doses employed were 5, 10, 20 and 50 kJ/m². Typically with a fluence rate of 150 W/cm² and a distance of 30 cm, the irradiation time was between 12 seconds to 2 min for all the doses used. At natural sunlight exposure level, these doses would be between 1.5 to 15 min at sea level (Pourzand et al, 1999). The higher doses of 20 and 50 kJ/m² would be more relevant for skin therapeutic purposes. This is because of the attenuation of the applied UVA dose by passage through the outer skin layers. The UVA doses were measured using an IL1700 radiometer (International light, Newbury, MA).

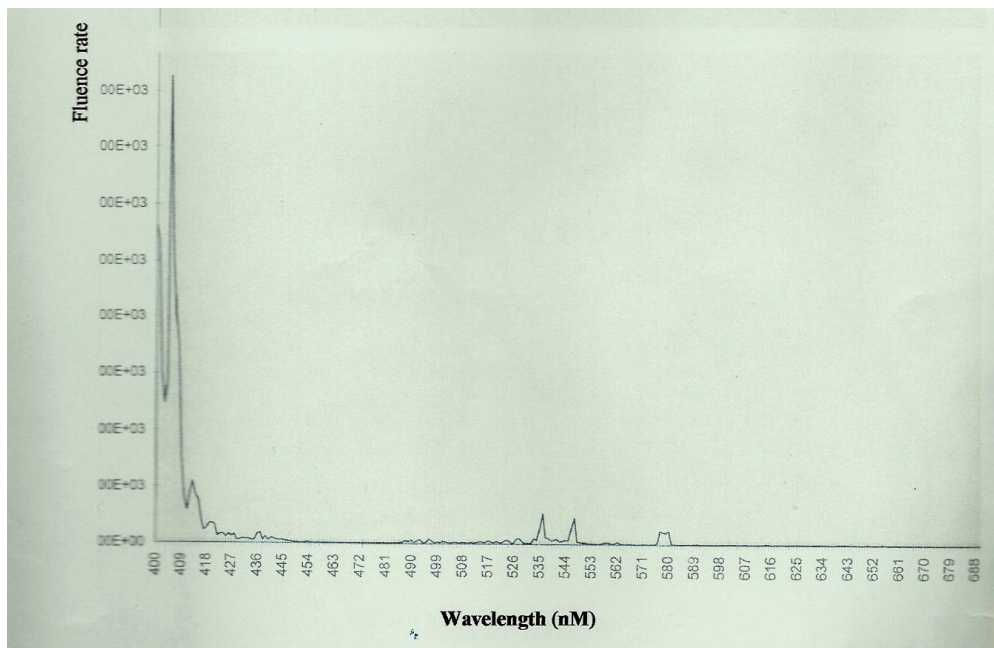
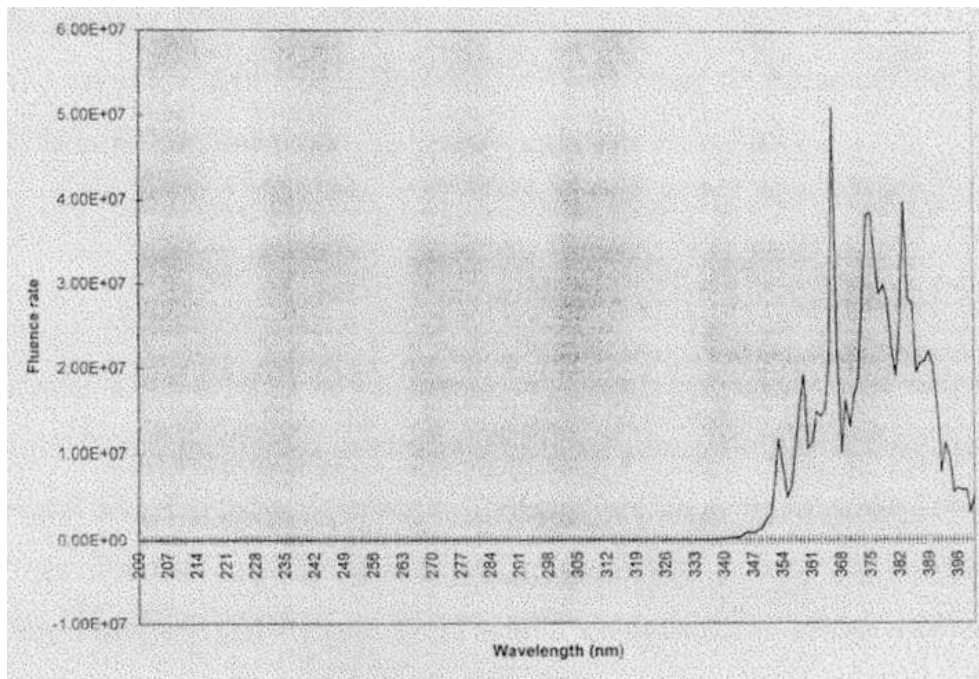


Figure 2.1. Emission spectrum from the Sellas 4kW UVA lamp

2.3.1.3 Irradiation procedure

Irradiation was carried out in a light-controlled air-conditioned room at 18°C in order to maintain the temperature of the cells to approximately 25°C throughout the radiation procedure. Prior to irradiation, cells were washed with PBS, followed by addition of PBS containing 5 ppm Ca^{2+} and Mg^{2+} to the cells in 3 cm plates to maintain cell membrane integrity. The cells were then irradiated with UVA doses of 5, 10, 20 or 50 kJ/m^2 . After irradiation, cells were incubated in conditioned medium (the retained medium in which the cells had been grown) prior to MTT or flow cytometry analyses. For split dose experiments, following the first irradiation cells were incubated for one hour in conditioned media under dark conditions at 37°C and then re-irradiated (or not), with UVA-doses of 5, 10, 20 and 50 kJ/m^2 in PBS supplemented with $\text{Ca}^{2+}/\text{Mg}^{2+}$. For ALA treated cells, cells were kept under dark condition prior to irradiation and then split dose irradiations, during the 1 h dark interval. The control samples were treated in the same manner except that they were not irradiated (i.e. kept in dark).

2.3.2. ALA treatment

ALA stock solution: 100 mM in H_2O

Chemical name: 5-aminolaevulinic acid hydrochloride.

Treatment: Cells were incubated with 0.5 mM ALA for 2 h (as previously established in this laboratory, Pourzand et al, 1990b) in 1% FCS DMEM at 37°C under dark conditions prior to irradiation.

2.3.3. Hemin treatment

Hemin stock solution: 20 mM hemin in dimethyl sulfoxide (DMSO)

Chemical name: Hemin chloride.

Treatment: Cells were treated with hemin (at a final concentration 10 μ M as previously established in this laboratory for the purpose of this study., Zhong, PhD thesis, 2000) for 18h at 37°C (5% CO₂) under dark conditions.

2.4. MTT Assay

2.4.1. Principle of the assay

Cellular dehydrogenase enzyme converts MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide], a yellow water-soluble substrate, into a dark blue formazan product that is insoluble in water. The amount of formazan produced is directly proportional to cellular reductive capacity and subsequently cell viability. So the MTT colorimetric assay (Mosmann, 1984; Berridge *et al*, 1996) can be used as a cell viability assay.

2.4.2. MTT stock solution

The MTT stock solution was prepared at a final concentration of 5 mg/ml in PBS. The solution was then filtered through a 0.22 μ m filter for sterilization and stored in small aliquots at -20°C.

2.4.3. Procedure

On the day of the experiment, MTT stock solution was first diluted in serum-free DMEM medium (SFM) to the final concentration of 0.5 mg/ml (i.e. MTT/SFM solution). In UVA-

based experiments, following UVA irradiation, the cells were first incubated for 30 min in conditioned media to recover. Then the conditioned media was replaced by MTT/SFM solution. Typically, 0.5 ml of MTT/SFM was added into each 3 cm plate and the cells were then incubated for 3h at 37°C in the CO₂ incubator under dark conditions. The MTT/SFM solution was then aspirated and 0.5 ml DMSO was added to each well. The plates were then swirled for 3 min on a 3D rocking platform (Stuart Scientific, UK). A small aliquot (i.e. usually 100 µl) was transferred from each plate to a 96 well plate and read by Dynatech plate reader MR-5000 (Dynatech, Guernsey, Channel Islands) at 570 nm using DMSO as a blank control. The results were expressed as percentage cell viability of relevant controls.

Figure 2.2 provides the summary of various treatments used prior to MTT assay.

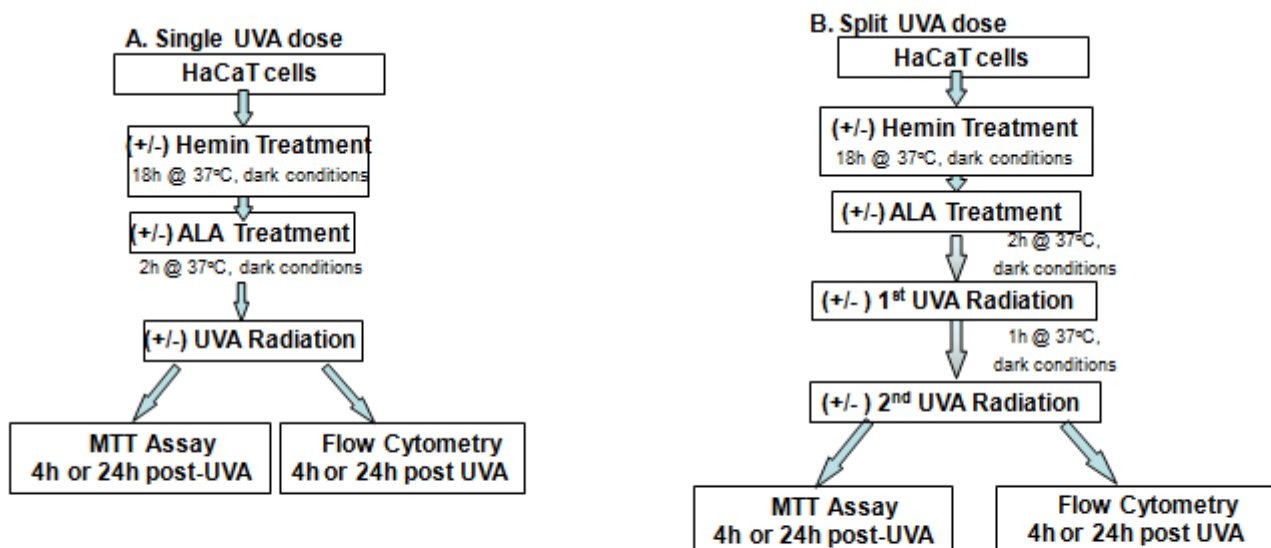


Figure 2.2: Experimental outline for UVA-ALA PDT single and split dose

strategies (+/- hemin).

2.5. Flow Cytometry

2.5.1. Principle of the assay

Quantification of apoptosis, by Annexin V-Fluorescein isothiocyanate (FITC) staining, and necrosis, by propidium iodide (PI) uptake, was evaluated by flow cytometry. In the early stages of apoptosis, phosphatidylserine (PS) translocates from the inner part of the plasma membrane to the outer layer. Annexin-V is a phospholipid-binding protein with a high affinity for PS. Identification of cell surface PS with Annexin-V serves as a marker for apoptotic cells. On the other hand necrotic cells that lose cell membrane integrity are stained with both PI and Annexin-V. Thus, Annexin-V and PI double staining can differentiate between necrotic and apoptotic cells (Zhong et al, 2004). In the present study, this assay was used to back up some of the key data obtained with the MTT assay. **Figure 2.2** provides the summary of various treatments used prior to flow cytometry assay.

2.5.2. Procedure

Following the various treatments, cells were washed with PBS and incubated in incubation buffer (10 mM Hepes / NaOH, pH 7.4, 140 mM NaCl, 5 mM CaCl₂) containing Annexin-V (20 µl/ml) and PI (20 µl/ml) for 15 min. Double stained cells were counted as necrotic whereas Annexin-V positive, PI negative cells were scored as apoptotic. A minimum of 10,000 cells were analyzed per sample on a BD FACSCanto™ flow cytometer (Becton Dickinson, Erembodegen, Belgium). The following settings were used for the analyzed cell line (HaCaT): FL1 (530 +/- 15 nm) in log data collection and FL3 in log data collection. Two dimensional dot plots were used to analyse the data of FL3 versus FL1 fluorescent profiles.

CellQuest software (Becton-Dickinson, Eremodegem, Belgium) was used to analyse the data with a dual parameter of FL1 (Annexin-V) and FL3 (PI).

2.6. Statistical analysis

Results are expressed as mean +/- standard deviation (SD). Data were analysed using paired t-tests. The *p* value of less than 0.05 was considered to be significantly different between groups of data.

CHAPTER THREE

RESULTS

3.1. Background

The use of UVA radiation for ALA-PDT of superficial skin lesions such as AK may be advantageous as UVA is more damaging than visible light. Indeed UVA radiation is a strong oxidizing agent that generates ROS in exposed cells. Previous studies from this laboratory have highlighted that in addition to ROS, UVA exposure also promotes an immediate increase in the intracellular level of LI in skin cells that acts as a catalyst to produce more harmful ROS which readily damage the cellular compartments, leading to cell death (Pourzand et al, 1999; Zhong et al, 2004; Reelfs et al, 2010). The UVA-induced LI release is a major factor in inducing damage to skin because the excess LI persists up to 2h after irradiation due to lack of storage as a result of UVA-mediated degradation of the iron storage protein, Ft. The presence of potentially harmful LI within the early hours after UVA irradiation has been shown to further exacerbate the peroxidative skin damage leading to necrotic cell death, since the excess LI can not be safely sequestered in Ft (Zhong et al, 2004; Reelfs et al, 2004; Yiakouvaki et al, 2006; Reelfs et al, 2010). This property of UVA could potentially be exploited advantageously to improve the efficiency of ALA-PDT of skin lesions such as AK. Indeed by using UVA as the light source and by fractionating the dose applied into two short pulses with 1h interval, the ALA-treated lesions could be further sensitized to UVA-induced damage since the first dose of UVA would trigger the formation of ROS and extensive release of LI which in turn would intensify the damage caused by the second challenge dose of UVA.

In the present study, we evaluated the response of spontaneously immortalized HaCaT keratinocytes to either single or two short pulses of UVA with a 1 h interval. The level of cell survival following various conditions was evaluated with the MTT viability assay. The key data were further verified by dual Annexin V/propidium iodide assay by flow cytometry.

3.2. The effect of ALA on HaCaT cell survival following irradiation with single doses of UVA

The preliminary data from this laboratory have previously indicated that a 2h treatment with ALA concentration of 0.5 mM could significantly sensitise the HaCaT keratinocytes to the damaging effects of UVA (unpublished data, Pourzand's laboratory, personal communication). To confirm these data and to evaluate the range of low doses of UVA that may be suitable for the present project, the HaCaT cells were treated with 0.5 mM ALA for 2 h and then exposed to a range of low doses of UVA (*i.e.* 5, 10, 20 and 50 kJ/m²). The MTT analysis was performed 24 h after irradiation. The results (**Figure 3.1**) revealed that in absence of ALA, low doses of UVA had no significant effect on cell viability when compared to untreated controls. However ALA treatment substantially increased the susceptibility of HaCaT cells to UVA-induced damage as upon UVA exposure, the percentage of cell viability in ALA-treated cells significantly decreased when compared to unirradiated controls.

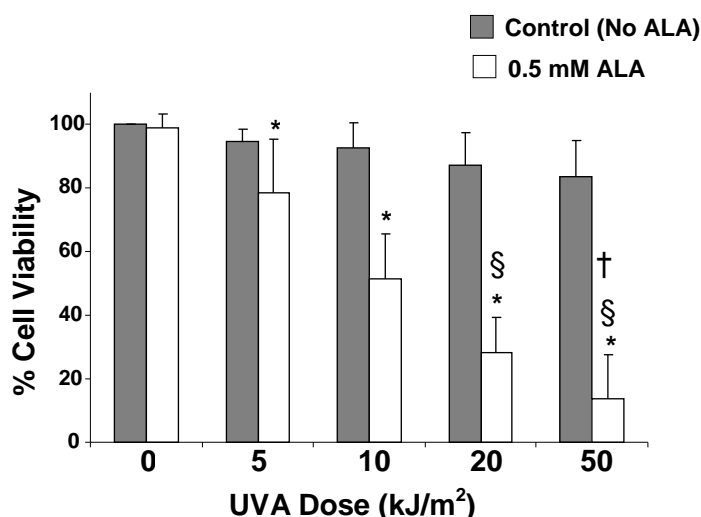


Figure 3.1. Effect of ALA on cell viability 24h after irradiation of cells with single doses of UVA.

HaCaT cells were treated with 0.5 mM ALA for 2h at 37°C under dark condition prior to irradiation with single UVA doses of 5, 10, 20 or 50 kJ/m². Control cells were treated the same except that they were either not irradiated (i.e. 0) and (or) not treated with ALA (i.e. grey columns).

MTT analysis was performed 24h after irradiation. The results were expressed as mean \pm standard deviation (n=3-17).

*: $p < 0.05$, significantly different from the unirradiated ALA-treated cells.

§: $p < 0.05$, significantly different from the ALA-treated cells irradiated with single dose of 10/0 kJ/m².

†: $p < 0.05$, significantly different from the ALA-treated cells irradiated with single dose of 20/0 kJ/m².

To confirm the results obtained with MTT, the level of apoptotic and necrotic cell death was scored by flow cytometry using the dual Annexin V/PI staining method. For this purpose, the ALA-treated cells were exposed to UVA doses of 10 or 50 kJ/m² and then incubated for 24 h at 37°C prior to analysis by flow cytometry. **Figure 3.2** is a representative example of such an analysis. The flow cytometry analysis showed residual apoptotic cells in some of the conditions used in this study. This is in agreement with previous findings from this laboratory showing that apoptosis is not the primary mode of cell death in HaCaT cells using these dosing parameters (Zhong *et al*, 2004). Indeed in control cells and in cells irradiated with

UVA alone or ALA alone, a low percentage of necrotic cell death was observed. But most importantly, ALA-treatment combined with UVA irradiation caused a dose-dependent increase in the percentage of necrotic cell death in HaCaT cells. The observed dose-dependent increase in necrotic cell death in combined ALA+UVA-treated cells in the flow cytometry analysis correlated with a reciprocal decrease in percentage of cell viability of the same conditions in MTT assay.

Taken together, the flow cytometry analysis was in agreement with the MTT analysis, as both assays confirmed that ALA-treatment strongly sensitises the HaCaT cells to UVA-induced cell damage and death. Furthermore, it was apparent that necrosis is the primary mode of cell death induced by UVA in ALA-treated cells.

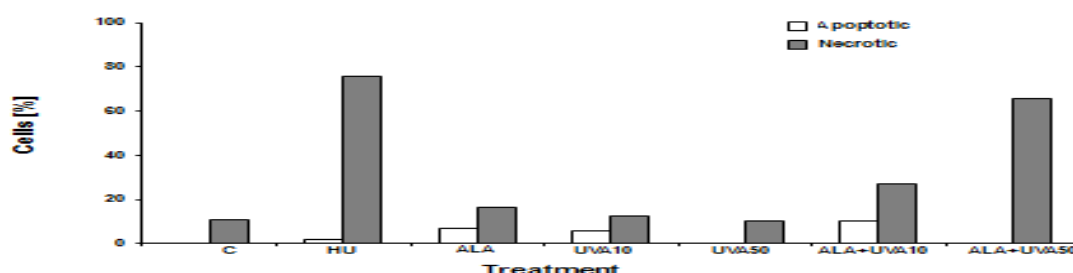


Figure 3.2. The effect of ALA treatment on the percentage of necrosis and apoptosis in HaCaT

cells 24h following UVA irradiation. HaCaT cells were treated with 0.5 mM ALA for 2h at 37°C under dark condition prior to irradiation with single doses of 10 or 50 kJ/m². Control cells were treated the same except that they were either not irradiated and (or) not treated with ALA. Flow cytometry analysis was performed 24h after the UVA irradiation following Annexin V-PI staining. ‘C’ refers to the untreated control; ‘HU’ is a positive control for necrosis where HaCaT cells treated with 20 μM hemin for 18h were irradiated with a UVA dose of 250 kJ/m²; ‘ALA’ refers to the cells treated with ALA alone; ‘UVA10’ and ‘UVA50’ refer to cells irradiated with UVA single doses of 10 and 50 kJ/m², respectively; ‘ALA+UVA10’ and ‘ALA+UVA50’ refer to ALA-treated cells irradiated with UVA single doses of 10 and 50 kJ/m², respectively. (n=2)

3.3. The effect of ALA on HaCaT cell survival following irradiation with a second dose of UVA

To determine whether a second challenge dose of UVA 1h following the first irradiation could further exacerbate the UVA-mediated cell damage of ALA-treated HaCaT cells, a series of UVA split-dose combination experiments were designed where cells were subjected to two short pulses of UVA with 1 h interval. To this regard, the ALA-treated HaCaT cells were first irradiated with a single dose of 5, 10, 20 or 50 kJ/m² UVA and then subjected to a second dose of 5, 10, 20 or 50 kJ/m², 1h following the first UVA radiation. The MTT viability assay was performed 24 h following the second UVA irradiation.

Figure 3.3 shows the summary of the first set of UVA split dose combination experiments using a second UVA dose of 5 kJ/m². As it can be seen, while a low single UVA dose of 5 kJ/m² only marginally decreased the cell viability of ALA-treated HaCaT cells, a second dose of the same intensity significantly decreased the ALA-treated cells' viability to 54.7% when compared to untreated control. Similarly, a second dose of 5 kJ/m² significantly decreased the viability of ALA-treated cells irradiated with an initial dose of 10 kJ/m² from 57% to 12%.

Furthermore, in ALA-treated cells irradiated with initial higher UVA doses of 20 or 50 kJ/m², the second dose of 5 kJ/m² also provided additional significant cell killing, when compared to ALA-treated cells irradiated with the corresponding single doses.

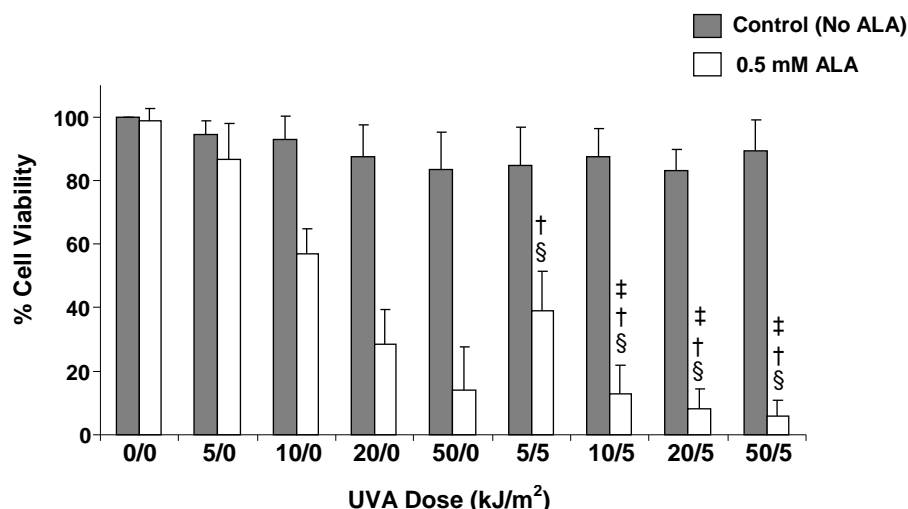


Figure 3.3. The effect of ALA on cell viability 24 h after the irradiation of cells with single or double split doses of UVA. HaCaT cells were first treated with 0.5 mM ALA for 2 h at 37°C under dark condition prior to irradiation with a single dose of 0, 5, 10, 20 or 50 kJ/m² (indicated as 5/0, 10/0, 20/0 and 50/0 respectively). For split-dose conditions, following the first irradiation cells were incubated for 1h in conditioned media and then exposed to a second UVA dose of 5 kJ/m² (indicated as 5/5, 10/5, 20/5 and 50/5 respectively). All control cells were treated the same except that they were either not irradiated (*i.e.* 0/0) and (or) not treated with ALA. MTT analysis was performed 24 h after the second irradiation.

The results were expressed as mean \pm standard deviation (n=3-17).

§: p<0.05, significantly different from the ALA treated cells irradiated with UVA single dose of 5/0 kJ/m².

†: p<0.05, significantly different from the ALA treated cells irradiated with UVA single dose of 10/0 kJ/m²

‡: p<0.05, significantly different from the ALA treated cells irradiated with UVA double dose of 5/5 kJ/m²

Figure 3.4 shows the summary of the second set of UVA split dose combination experiments using a second UVA dose of 10 kJ/m². As it can be seen, compared to single dose treatment (*i.e.* 10/0 +ALA), a second UVA dose of 10 kJ/m² significantly decreased the cell viability of ALA-treated cells pre-irradiated with a UVA dose of 10 kJ/m² (*i.e.* from 57.1 \pm 7.4 to 17.1 \pm 9.4). Similarly, compared to single dose treatment (*i.e.* 20/0 +ALA), this second dose

triggered additional decrease in cell viability in ALA-treated cells pre-irradiated with a moderate dose of 20 kJ/m². Finally, in this set of split-dose combination, the ‘50/10+ALA’ condition provided the most efficient decrease in percentage cell viability that was not only significantly lower than all the ALA-treated cells irradiated with single UVA dose but was also lower than the ALA-treated ‘10/10’ double-dose combination. Also a significant decrease was observed in the control cells irradiated with double UVA dose of 50/10 in comparison to its corresponding single UVA dose of 50, which was of interest.

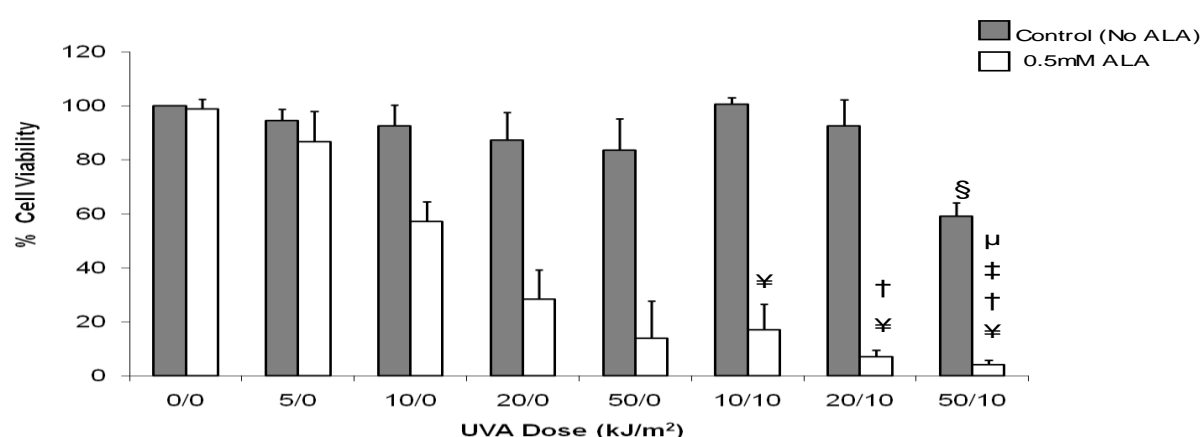


Figure 3.4. Effect of ALA on cell viability 24h after the irradiation of cells with single or double dose of UVA. HaCaT cells were first treated with 0.5 mM ALA for 2h at 37°C under dark condition prior to irradiation with a single dose of 0, 10, 20 or 50 kJ/m² (indicated as 10/0, 20/0 and 50/0 respectively). For double dose conditions, following the first irradiation cells were incubated for 1h in conditioned media and then exposed to a second UVA dose of 10 kJ/m² (indicated as 10/10, 20/10 and 50/10 respectively). All control cells were treated the same except that they were either not irradiated (i.e. 0/0) and (or) not treated with ALA. The MTT analysis was performed 24h after the second irradiation. The results were expressed as mean ± standard deviation (n=3-17).

¥: p<0.05, significantly different from the ALA treated cells irradiated with UVA single dose of 10/0 kJ/m².

†: p<0.05, significantly different from the ALA treated cells irradiated with UVA single dose of 20/0 kJ/m².

‡: p<0.05, significantly different from the ALA treated cells irradiated with UVA single dose of 50/0 kJ/m².

μ: p<0.05, significantly different from the ALA treated cells irradiated with double UVA dose of 10/10 kJ/m².

§: p<0.05, significantly different from the control cells irradiated with single UVA dose of 50/0 kJ/m².

To confirm the effect of split-dose UVA treatments in improving the efficiency of cell killing of ALA-treated HaCaT cells, the level of apoptotic and necrotic cell death was scored by flow cytometry using the dual Annexin V/PI staining method. For this purpose, the ALA-treated cells were exposed either to single or double split-dose of 10 kJ/m² UVA with 1h interval and then incubated for 24h at 37°C prior to analysis by flow cytometry. As it can be seen in **Figure 3.5**, compared to single dose treatment (ALA+UVA10), a second UVA dose of 10 kJ/m² 1h following the initial dose, increased the percentage of necrotic cell death in ALA-treated cells (i.e. from 17% in 'ALA+UVA10' to 50% in 'ALA+UVA10+UVA10' condition). However in the control groups (i.e. in 'C' and 'ALA') and in cells irradiated with UVA alone (i.e. 'UVA10'), the percentage of necrotic cell death remained very low. The observed increase in necrotic cell death in 'ALA+UVA10+UVA10' condition in flow cytometry analysis correlated with a reciprocal decrease in percentage cell viability of the same condition in MTT assay.

Taken together, the flow cytometry analysis was in agreement with the MTT analysis, as both assays confirmed that two short pulses of UVA with 1h interval further sensitises the ALA-treated HaCaT cells to UVA-induced cell damage and death. Furthermore it was again apparent that necrosis is the primary mode of cell death induced by UVA in ALA-treated cells.

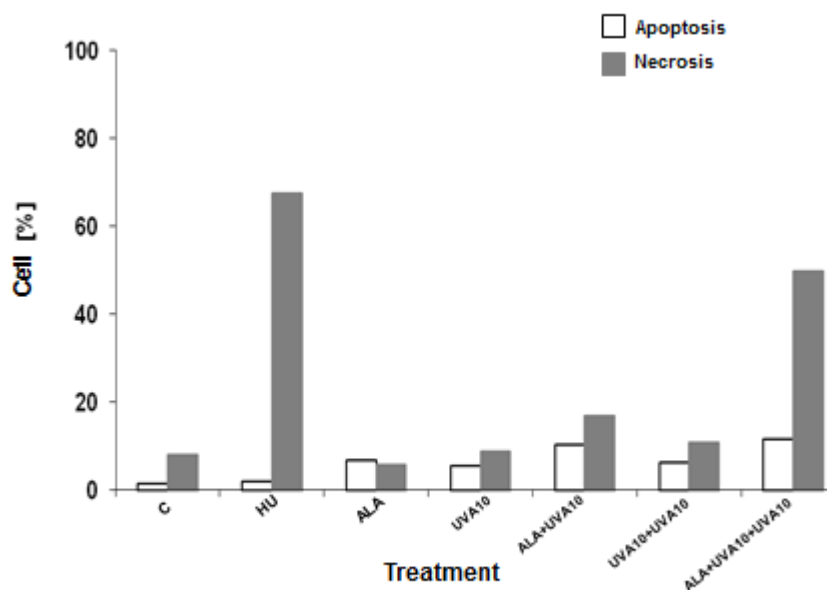


Figure 3.5. The effect of ALA on the percentage of necrosis and apoptosis 24 h after irradiation of cells with single or double split doses of UVA. HaCaT cells were first treated with 0.5 mM ALA for 2 h at 37°C under dark condition prior to irradiation with a single dose of 10 kJ/m² (indicated as ‘ALA+UVA10’). For split dose conditions, following the first irradiation cells were incubated for 1h in conditioned media and then exposed to a second UVA dose of 10 kJ/m² (shown as ALA+UVA10+UVA10). All control cells were treated the same except that they were either not irradiated (i.e. ‘C’ and ‘ALA’) and (or) not treated with ALA (i.e. ‘UVA10’ and ‘UVA10+UVA10’). ‘HU’ is a positive control for necrosis where HaCaT cells pre-treated with 20 µM hemin for 18 h were irradiated with a UVA dose of 250 kJ/m². Flow cytometry analysis was performed 24 h after the UVA irradiation following Annexin-V+PI staining.

Figure 3.6 shows the summary of the third set of UVA split dose combination experiments using a second UVA dose of 20 or 50 kJ/m². As it can be seen, the percentage cell viability in ALA-treated cells irradiated with two split-dose of 20 or 50 kJ/m² was significantly lower than ALA-treated cells irradiated with the corresponding single UVA doses. Interestingly in

ALA-treated cells, the decrease in percentage cell viability of cells with double UVA dose combination of '50/20' kJ/m² was similar to that '50 /50' kJ/m² (n = 2).

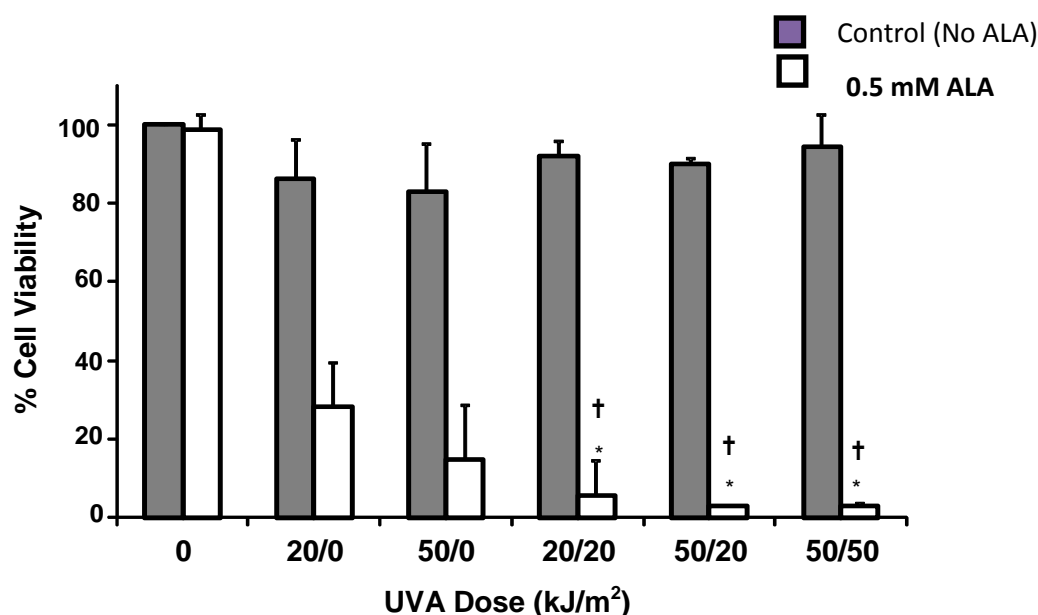


Figure 3.6. Effect of ALA on cell viability 24 h after the irradiation of cells with single or double dose of UVA. HaCaT cells were first treated with 0.5 mM ALA for 2 h at 37°C under dark condition prior to irradiation with a single dose of 20 or 50 kJ/m² (indicated as 20/0 and 50/0, respectively). For double dose conditions, cells were incubated for 1 h in conditioned media before irradiation with second doses of 20 and 50 kJ/m² (shown as 20/20, 50/20 and 50/50 respectively). All control cells were treated the same except that they were either not irradiated (i.e. 0/0) and (or) not treated with ALA. MTT analysis was performed 24 h after the second irradiation. The results were expressed as mean ± standard deviation (n=3-17).

*: p<0.05, significantly different from the ALA treated cells irradiated with UVA single dose of 20/0 kJ/m².

†: p<0.05, significantly different from the ALA treated cells irradiated with UVA single dose of 50/0 kJ/m².

Figure 3.7 shows the summary of the fourth set of UVA split dose combination experiments using a first UVA dose of 5 kJ/m². As it can be seen, the combination of low first dose of UVA with a higher second dose is less efficient than previous sets of split-dose combination

experiment and was no better than a single dose of ALA +20 kJ/m² UVA unless a second UVA dose of 50 kJ/m² was employed. This is presumably because the first low UVA insult does not provide enough impact in terms of radiation-induced ROS production and LI release to influence the extent of damage caused by second irradiation and as a result even higher second doses of UVA fails to provide the desired cumulative cell damage. These results also demonstrate that when ALA-treated cells are initially irradiated with a low dose of 5 kJ/m², the second UVA dose challenge of 5, 10 or 20 kJ/m² fails to provide a dose-response. Furthermore no significant difference in cell viability could be observed between ALA-treated cells irradiated with single UVA doses of 20 and 50 kJ/m² and ALA-treated double UVA dose combination of '5/20' and '5/50' kJ/m². Nevertheless the double-dose combinations of 5/5 and 5/10 kJ/m² significantly decreased the viability of ALA-treated cells when compared to ALA-treated cells irradiated with single dose of 5 or 10 kJ/m². Overall, in comparison with previous sets of combination, the results of **Figure 3.7** strongly suggest that using such a low initial dose of UVA is not an efficient approach to improve the efficiency of cell killing with UVA split-dose ALA-PDT.

Based on these experiments, it was concluded that the third UVA split dose combination (i.e. results illustrated in **Figure 3.6**) provide the most promising approach to improve the efficiency of cell killing of ALA-treated HaCaT cells, since the initial UVA insult appears to be high enough to exacerbate the damage caused by the second dose challenge of UVA.

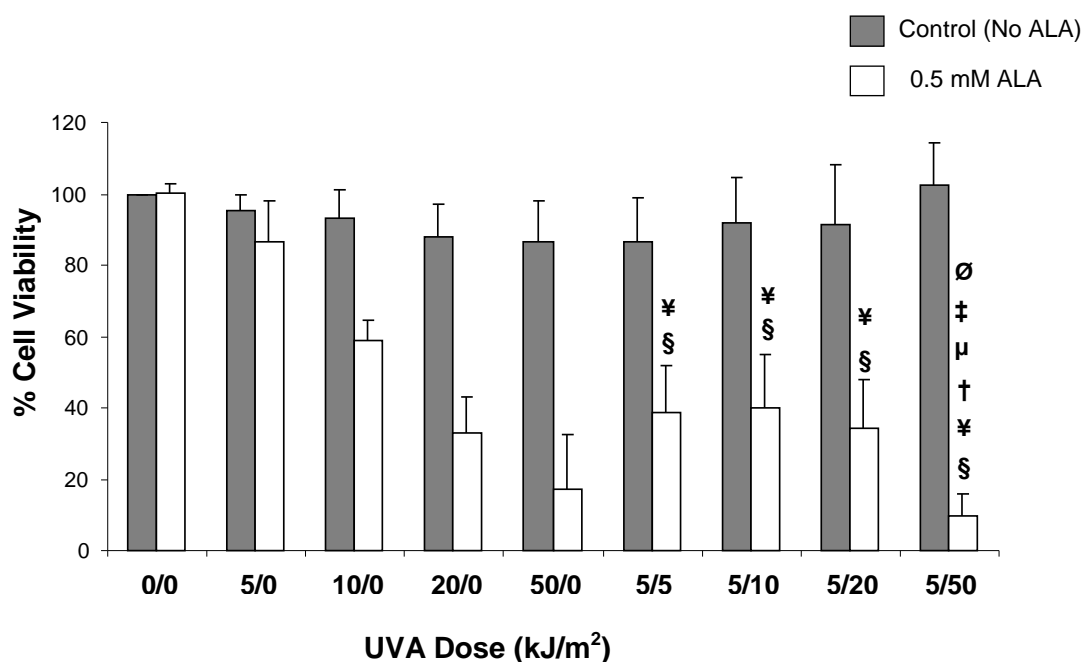


Figure 3.7. Effect of ALA on cell viability 24h after the irradiation of cells with single or double dose of UVA. HaCaT cells were first treated with 0.5 mM ALA for 2h at 37°C under dark condition prior to irradiation with a single dose of 0, 5, 10, 20 or 50 kJ/m² (indicated as 5/0, 10/0, 20/0 and 50/0). For double dose conditions, following the first irradiation, cells were incubated for 1h in conditioned media and then exposed to second UVA doses of 5, 10, 20 or 50 kJ/m² (shown as 5/5, 5/10, 5/20 and 5/50 respectively). All control cells were treated the same except that they were either not irradiated (i.e. 0/0) and (or) not treated with ALA. MTT analysis was performed 24h after the irradiation. The results were expressed as mean ± standard deviation (n=3-17).

§: p<0.05, significantly different from the ALA treated cells irradiated with UVA single dose of 5/0 kJ/m².

¥: p<0.05, significantly different from the ALA treated cells irradiated with UVA single dose of 10/0 kJ/m².

‡: p<0.05, significantly different from the ALA treated cells irradiated with UVA single dose of 20/0 kJ/m².

μ: p<0.05, significantly different from the ALA treated cells irradiated with UVA double dose of 5/5 kJ/m².

‡: p<0.05, significantly different from the ALA treated cells irradiated with UVA double dose of 5/10 kJ/m².

Ø: p<0.05, significantly different from the ALA treated cells irradiated with UVA double dose of 5/20 kJ/m².

3.4. The effect of hemin and ALA on HaCaT cell survival following irradiation with single or double split-doses of UVA

Previous data from this laboratory have demonstrated that Ft iron is the major source of LI release that is observed immediately following the radiation insult (Pourzand *et al*, 1999; Zhong *et al*, 2004; Reelfs *et al*, 2010). Indeed preventing the UVA-mediated Ft degradation by specific protease inhibitors substantially decreased the UVA-induced increase in LIP (Pourzand *et al*, 1999). Studies by Zhong *et al* (2004) further demonstrated that iron loading of cultured skin fibroblasts and keratinocytes with overnight hemin treatment (i.e. 10-20 μM) can lead to a 4-5 fold increase in the intracellular level of Ft that could in turn cause a massive increase in LI (i.e. 3-4 fold increase) following UVA irradiation. The higher UVA-induced LI release following hemin treatment strongly sensitises the skin keratinocyte cells to UVA-induced oxidative damage leading to early necrotic cell death 4 h following radiation insult (Zhong *et al*, 2004; Reelfs *et al*, 2010).

To determine the critical role of UVA-induced LI release in exacerbating the UVA-induced damage in ALA-treated cells, HaCaT cells were first treated for 18 h with the previously established concentration of 10 μM hemin (Zhong, 2002) and then subjected to UVA doses of 5, 10 and 20 kJ/m^2 following a 2 h treatment with ALA. The MTT analysis was performed 4 h following the UVA irradiation. The results (**Figure 3.8**) demonstrated that hemin treatment *per se* or combined with UVA doses of 5-20 kJ/m^2 had no significant effect on HaCaT cells' viability. Furthermore the sensitivity of ALA-treated cells to UVA was less apparent with a light dose of 5 kJ/m^2 . Nevertheless hemin treatment strongly sensitised the ALA-treated cells to UVA doses of 10 and 20 kJ/m^2 in a dose-dependent manner. These results are in agreement with previous findings from Zhong *et al* (2004) and are consistent

with the notion that increasing UVA-induced iron release by hemin treatment exacerbates the damaging effect of UVA in photosensitised ALA-treated cells.

The low UVA dose of 5 kJ/m² failed to show significant damage in hemin+ALA treated cells, presumably because this dose was not high enough to promote the breakdown of highly accumulated Ft molecules and to release their potentially harmful LI.

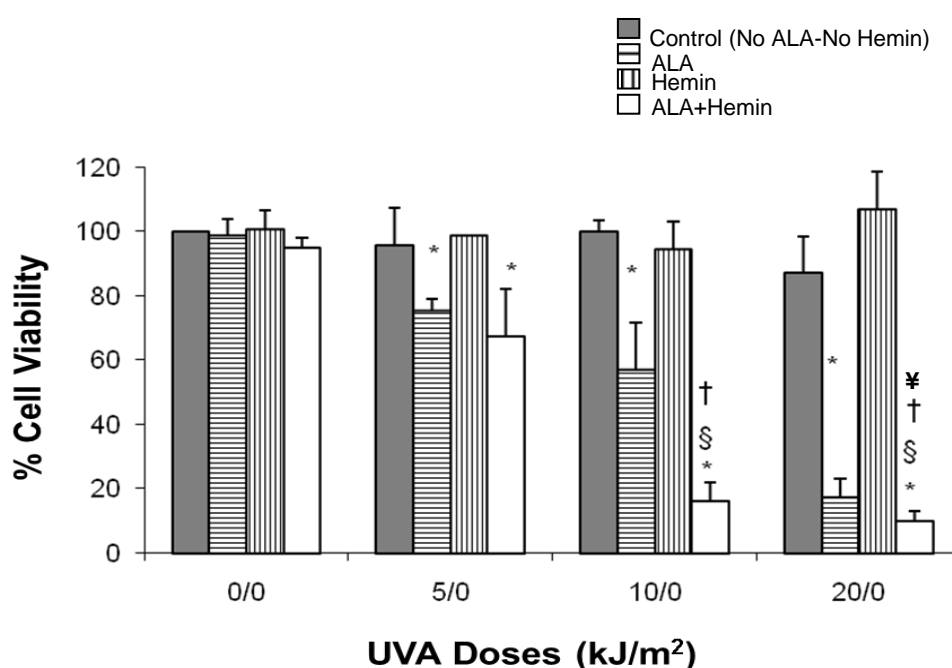


Fig 3.8. Effect of ALA and (or) hemin on cell viability 4h after irradiation of cells with single doses of UVA. HaCaT cells were first treated (or not) with 10 µM hemin for 18h and then incubated (or not) with 0.5 mM ALA for 2h at 37°C under dark condition prior to irradiation with UVA doses 5, 10, or 10 kJ/m². All control cells were treated the same except that they were either not irradiated and (or) not treated with ALA and hemin. MTT analysis was performed 4h after UVA irradiation. The results were expressed as mean ± standard deviation (n=3-5).

*: p<0.05, significantly different from the unirradiated ALA-treated cells.

§: p<0.05, significantly different from the corresponding irradiated ALA-treated cells.

†: p<0.05, significantly different from the Hemin+ALA treated cells irradiated with UVA single dose of 5 kJ/m².

¥: p<0.05, significantly different from the Hemin+ALA treated cells irradiated with UVA single dose of 10 kJ/m².

To confirm the effect of hemin treatment observed in **Figure 3.8** with MTT, the level of apoptotic and necrotic cell death was scored by flow cytometry using the dual Annexin V/PI staining method 4 h following irradiation with a UVA dose of 10 kJ/m². As it can be seen in **Figure 3.9**, compared to cells treated with ALA alone, hemin pre-treatment provoked substantial necrotic cell death in ALA-treated HaCaT cells even at this early time point after irradiation. These results are in agreement with MTT assay and are consistent with the notion that increasing UVA-induced LI release by hemin treatment strongly sensitises the ALA-treated cells to iron-mediated oxidative cell killing. Furthermore it was again apparent that necrosis is the primary mode of cell death induced by UVA in hemin +/-ALA-treated cells using these particular dosing parameters.

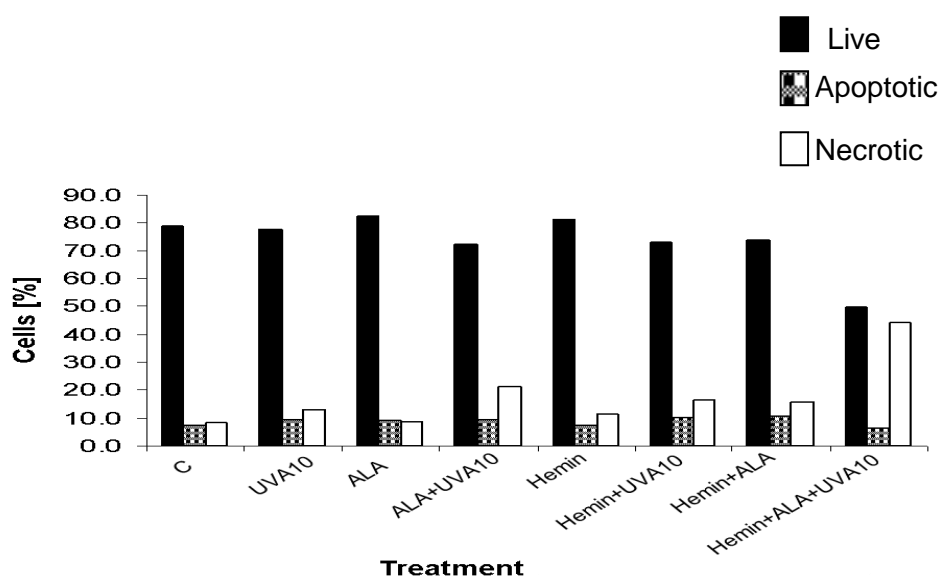


Figure 3.9. The effect of hemin and ALA treatments on the percentage of necrosis and apoptosis in HaCaT cell line. HaCaT cells were first treated (or not) with 10 µM hemin for 18 h and then incubated (or not) for 2 h with 0.5 mM ALA at 37°C under dark condition prior to irradiation with a single UVA dose of 10 kJ/m². All control cells were treated the same except that they were either not irradiated and (or) not treated with ALA and hemin. Flow cytometry analysis was performed 24 h after the UVA irradiation following Annexin-V-PI staining.

To evaluate whether hemin treatment could also exacerbate the effect of second dose challenge of UVA in ALA-treated cells, the hemin+ALA-treated HaCaT cells were irradiated either with single UVA doses of 5 or 10 kJ/m² or with a double UVA dose combination of 10 and 5 kJ/m² with a 1 h interval. The MTT analysis was performed 4h following irradiation. The results (**Figure 3.10**) demonstrated that compared to hemin+ALA-treated cells irradiated with single UVA doses of 5 and 10kJ/m², the viability of hemin+ALA-treated cells further decreases following a second low UVA dose of 5kJ/m².

Overall, these results suggested that iron loading of cells with hemin prior to ALA+UVA treatment can not only increase the susceptibility of ALA-treated cells to low single doses of UVA but also could exacerbate the damage caused by an extremely low second dose of UVA, even within early hours after irradiation.

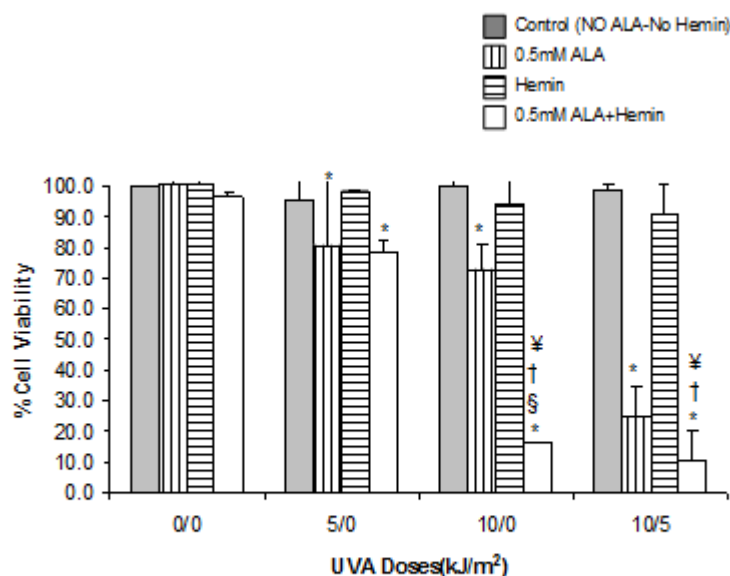


Figure 3.10. Effect of hemin on cell viability of ALA-treated cells 24h after irradiation with single or double split-doses of UVA. HaCaT cells were first treated (or not) with 10 μ M hemin for 18h and then incubated (or not) for 2h with 0.5 mM ALA at 37°C under dark condition prior to irradiation with single doses of 5 and 10 kJ/m². For split-dose conditions, following the first irradiation, cells were incubated for 1h in conditioned media and then exposed to a second UVA dose of 5 kJ/m².

All control cells were treated the same except that they were either not irradiated and (or) not treated with ALA and hemin. MTT analysis was performed 4h after UVA irradiation.

The results are expressed as mean \pm standard deviation (n=3-5).

*: $p < 0.05$, significantly different from the unirradiated ALA-treated cells.

§: $p < 0.05$, significantly difference from irradiated ALA-treated cells.

†: $p < 0.05$, significantly different from the hemin-treated cells irradiated with UVA single dose of 5/0 kJ/m².

¥: $p < 0.05$, significantly different from the hemin-treated cells irradiated with UVA single dose of 10/0 kJ/m².

CHAPTER FOUR

Discussion

Porphyrins and, in particular, PPIX are thought to be very important UVA chromophores, since they possess a band absorption with a peak around 405-410 nm that tails within the UVA range and have the potential of generating $^1\text{O}_2$ and other ROS upon UVA irradiation. Therefore for the treatment of readily accessible superficial skin lesions, ALA-PDT in the UVA region (320-400 nm) may be preferable to red or green wavebands, because of its higher efficiency. Indeed blue light in combination with ALA has been shown to be effective in the PDT of superficial skin lesions such as AK. Blue light that tails within the Soret band (410 nm) of PPIX is a powerful alternative light source to red light as it would give the largest cell inactivation down to about 2 mm from the surface in human skin and muscle tissues as well as in superficial BCC (Szeimies *et al*, 1995; Peng *et al*, 1997).

Although compared to red light, high doses of UVA have been shown to cause mutations in DNA (Kvam *et al*, 1997), it is thought that the proposed low doses in the present study are unlikely to be mutagenic. Nevertheless further experiments are required to prove this point.

Pourzand *et al* (1999b) have previously demonstrated that accumulating endogenous PPIX by exogenous ALA treatment strongly sensitises human skin fibroblasts to UVA photokilling (Pourzand *et al*, 1999b). A study by Buchczyk *et al* (2001) comparing red, green and UVA light sources in ALA-PDT of human skin cells has further demonstrated that compared with red light source, UVA-based ALA-PDT was 40-fold more potent in killing cultured human skin fibroblasts and still 10-fold more potent than ALA-PDT with green light. The high cytotoxicity of UVA-based ALA-PDT relied on the efficient formation of $^1\text{O}_2$ as was demonstrated with modulators of $^1\text{O}_2$ -half-life. In the present study, further evidence has been

provided that demonstrates that ALA-PDT with UVA is also effective in killing of human skin keratinocytes despite their high resistance to UVA-mediated oxidative damage and cell death (Pourzand and Tyrrell; 1999; Reelfs *et al*, 2010). Indeed previous studies from this laboratory have demonstrated that epidermal keratinocyte cells (including HaCaT) are resistant to up to high UVA doses of 750 kJ/m^2 (see Reelfs *et al*, 2010), but ALA treatment used in this study strongly sensitises the HaCaT cells to extremely low doses of UVA within the range of $10\text{-}100 \text{ kJ/m}^2$ (*i.e.* by taking into account both single and cumulative double UVA doses). The Sellas UVA light source used in this study has a significant peak at 405 nm that overlaps with the Soret band of PPIX that should almost certainly contribute to the efficient cell killing of ALA-treated HaCaT keratinocytes.

$^1\text{O}_2$ and H_2O_2 are thought to be the most important ROS generated intracellularly by UVA, promoting biological damage in exposed tissues *via* iron-catalysed oxidative reactions (Vile and Tyrrell, 1995). It has been shown that UVA doses at natural exposure level (*i.e.* $100\text{-}500 \text{ kJ/m}^2$) induce lipid peroxidation in membranes of human primary fibroblasts and keratinocytes via pathways involving iron and $^1\text{O}_2$ (Vile and Tyrrell, 1995; Morlier *et al*, 1991; Punnonen *et al*, 1991). The UVA-mediated increase in LIP has been shown to further exacerbate the peroxidative damage in cultured skin cells that may lead to the loss of cell membrane integrity and cell death (Zhong *et al*, 2004). It is anticipated that the damaging effect of UVA would become even more important in ALA-treated skin cells as the combination of artificially increased photochemical generation of $^1\text{O}_2$ and other ROS by endogenously accumulated PPIX along with UVA-mediated increase in intracellular LI could potentiate the formation of highly reactive oxygen species that would trigger substantial peroxidative membrane damage leading to necrotic cell death. Indeed, iron ‘at’ or ‘near’ strategic targets such as cell membranes, can undergo redox cycling by reacting sequentially

with one electron reductants and oxidants, thereby generating toxic oxidants such as $\cdot\text{OH}$ and lipid derived alkoxyl and peroxy radicals, can elicit biological damage and cell death (Aust *et al*, 1985; Halliwell and Gutteridge, 1992). Therefore the simultaneous presence of LI and ROS generated by UVA in ALA-treated cells could be effective in therapy of superficial skin lesions. Nevertheless the presence of excess labile iron could decrease the level of ALA induced PPIX accumulation via ferrochelatase dependent conversion of PPIX to haem. It is clear that in the future studies the correlation of iron accumulation by UVA and the level of conversion of PPIX to haem have to be investigated.

Previous studies from this laboratory have already demonstrated the impact of UVA-induced LI release in promoting necrotic cell death in skin fibroblasts. Furthermore, it has been demonstrated that iron loading of human keratinocytes with hemin or iron citrate substantially sensitises the human keratinocyte cells to UVA-induced necrotic cell death, as a result of UVA-induced increase in LIP. Indeed hemin treatment has been shown to provoke substantial necrosis in HaCaT cells irradiated with a low UVA dose of 100 kJ/m^2 , 1-4h following radiation treatment (Zhong *et al*, 2004). In the present study we further demonstrate that ALA treatment in combination with hemin further sensitises the keratinocytes to very low doses of UVA, presumably as a result of increase in UVA-induced LI release. The split-dose combination experiments carried out in the present project further reveal the importance of the first UVA dose to exacerbate the damage produced by the second UVA dose. Indeed the higher initial dose of UVA has a critical impact in determining the efficiency of cell killing following the second UVA dose, presumably because the level of first dose-mediated ROS generation and LI release directly contribute to the extent of damage caused by second UVA insult. Nevertheless future studies are necessary to measure the level of ROS production and LI released upon our experimental conditions.

Previous studies have demonstrated the importance of light fractionation in improving the efficiency of PDT of tumours (*e.g.* Curnow *et al*, 1999). Iron chelation has also been proposed as a mean to improve the efficiency of ALA-PDT and according to a comparative study carried out by Curnow and colleagues, both light dose fractionation and iron chelation, could significantly enhance the efficiency of ALA-PDT in the normal and neoplastic tissues investigated (Curnow *et al*, 2006).

Figure 4.1 illustrates our proposed mechanism of cell killing by single or split-dose UVA irradiation in combination with ALA +/- hemin, based on the results obtained in the present study. The main points of this proposal are discussed below:

- **Figure 4.1A:** In ALA-treated cells, the first dose of UVA not only triggers the formation of ROS but also promotes extensive release of LI as a result of Ft degradation which in turn sensitizes the cells to a second challenge dose of UVA resulting in higher necrotic cell death. This hypothesis is strengthened by our double split-dose combination studies showing that the extent of UVA-induced cell killing is dependent on the dose of the first UVA irradiation. When a low dose of 5 kJ/m^2 is applied first, the overall decrease in cell viability is moderate after the second irradiation even with higher UVA doses of 10 or 20 kJ/m^2 . The UVA dose of 50 kJ/m^2 on the other hand appears to be the only dose that has effective cytotoxicity in ALA-treated cells both as a single dose or in combination with other doses. Nevertheless our results demonstrated that lower double-dose UVA combinations of 20/5, 20/10 and 20/20 kJ/m^2 are also highly effective cytotoxic combinations in ALA-treated cells. Therefore the dose combination of 20/5 could be less time consuming in clinical practice.

- **Figure 4.1B:** In overnight hemin-treated cells, the Ft level is much higher than untreated cells (Zhong *et al*, 2004). So UVA-irradiation of hemin+ALA-treated cells would promote higher UVA-induced LI release which in turn in conjunction with generated ROS will further

exacerbate the susceptibility of the cells to UVA-induced oxidative damage leading to higher necrotic cell death. Although our pilot study performed in this one-year MPhil project is in agreement with this hypothesis, in future studies it is necessary to measure the LI released after UVA in ALA+/- hemin-treated cells to be able to show a direct correlation between the amount of LI release and damage caused.

Previous data from our laboratory have shown that hemin-mediated increase in Ft in HaCaT cells persists up to 48 h following treatment. This may be used as a potential intervention therapy to improve the efficiency of clinical ALA + UVA in clinical settings (Zhong *et al*, 2004).

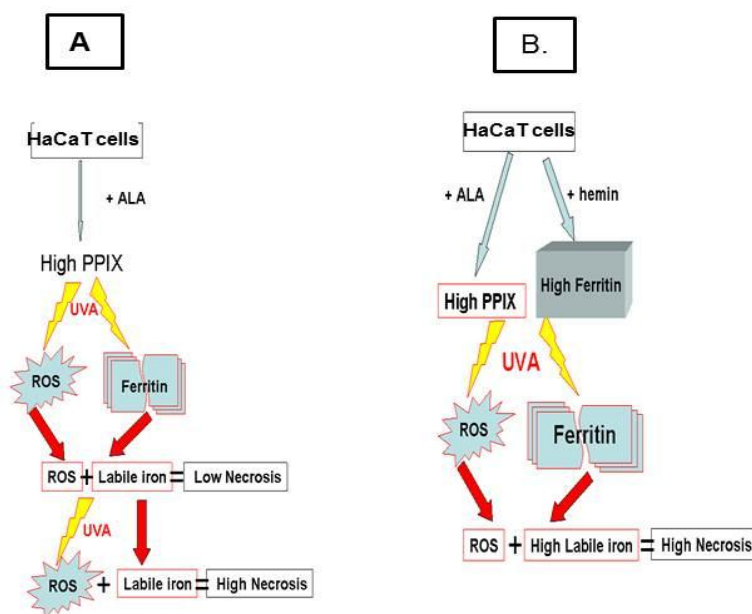


Figure 4.1. The proposed effect of UVA-induced iron release on ALA (+/-hemin)-treated HaCaT cells.

(A). Treatment of HaCaT cells with ALA promotes an increase in the level of endogenous photosensitiser PPIX that sensitises the cells to UVA irradiation leading to generation of ROS and labile iron release as a result of Ft degradation. The generated ROS along with labile iron increase by UVA provokes damage to cells leading to necrotic cell death. A second UVA dose of UVA, 1h following the first irradiation, triggers the formation of additional ROS that in conjunction with excess labile iron still present within cells promote further oxidative damage to HaCaT cells leading to higher necrotic cell death. However future studies are necessary to clarify the extent of contribution of UVA irradiation and/or accumulated PPIX to the observed cell death.

(B). Overnight hemin treatment of HaCaT cells promotes an increase in the level of intracellular Ft. ALA treatment of cells in the next day promotes an increase in the level of endogenous photosensitiser PPIX. The UVA irradiation of the hemin+ALA treated cells not only promotes the formation of ROS via PPIX but also leads to substantial increase in labile iron as a result of proteolytic degradation of highly accumulated Ft. This combination provokes substantial damage to cells leading to high necrotic cell death.

Topical ALA-PDT is an effective method for treating sun-damaged skin with multiple AK (Morton *et al*, 2002). The major side-effect of PDT is the pain experienced during the treatment (Morton *et al*, 2002). Management of treatment-related pain remains still a considerable challenge in patients (Morton *et al*, 2002). Further optimization of the light source, dose and period of treatment therefore seems crucial to alleviate pain. The pilot study carried out in this MPhil project provides strong evidence that applying short pulses of UVA radiation to ALA-treated skin cells is a fast and effective approach to promote cell death. Therefore applying split low doses of UVA radiation in combination with ALA may be used as an effective intervention strategy to decrease considerably the time of radiation treatment and therefore reduce the pain associated with prolonged conventional topical ALA-PDT of AK lesions using LED.

Our proposed methodology responds to the urgent need of finding alternative ways to alleviate ALA-PDT-related pain, by reducing to minimum the period of radiation. Indeed our results demonstrate that in ALA-treated skin cells, the low split- dose UVA radiation strategy provides effective result with 2 short pulses of 10-50 kJ/m². In a clinical setting, typically with a fluence rate of 150 W/cm², and the distance of 30 cm, the irradiation time with these UVA doses will be between 30 seconds to 2 min for all doses used. So such short treatments are likely to make the pain more tolerable. In comparison, for example in Bath Royal United Hospital, the MAL-PDT of AK is carried out with an LED (631 nm) light source for 9 min to achieve a dose of 37 J/cm² with a distance of 5-8 cm. The potentially effective and specially the fast damaging consequence of split low dose UVA protocol, when compared to prolonged LED radiation treatment, highlights our proposed strategy as a rapid and simple means to improve the effectiveness of conventional ALA-PDT of skin lesions while decreasing

considerably the time of irradiation that is directly related to the extent of pain endured by patients.

However, this treatment can only be suggested for the most superficial lesions and not for all currently licensed applications. Extensive follow ups however may be required to monitor the carcinogenic risk of the treatment. One drawback of the proposed treatment would be that the patients will spend more time in clinics and that would decrease the number of patients treated per day.

The work outlined here addresses further the suitability of UVA low split dose strategy as a possible therapy for epidermal diseases, including non-melanoma skin cancer (NMSC). Based on our pilot study presented in this MPhil work, the effectiveness of UVA split-dose strategy could be further tuned in the future studies using a series of cultured AK and SCC cell lines in both monolayer and 3-dimensional organotypic raft cultures. The best split dose protocol could then be evaluated in an open pilot study in collaboration with dermatologists looking at a few patients for instance with symmetrical AK lesions. The split dose UVA-ALA-PDT might have also a future in treatment of psoriasis, as at present the topical ALA-PDT clinical studies with psoriatic patients is often interrupted because of the severe pain experienced by patients. So decreasing the time of ALA-PDT with split-dose UVA strategy might provide a powerful alternative to present modalities. Psoralen with UVA has already been used for treatment of psoriasis but this treatment is accompanied with severe side effects. Short term side effects are increased photosensitivity and gastrointestinal and ocular side effects when administered orally, also redness, swelling and occasional blister formation, fever and general malaise. Long term side effects are promotional skin cancer (Honigsmann *et al*, 1982; Honigsmann *et al*, 2001; Cox *et al*, 1987). Furthermore while in general the PUVA therapy doses are between 5-10 kJ/m², because of the necessity of repeated therapies, the cumulative UVA doses in PUVA could reach values up to 250-500

kJ/m^2 that is detrimental to patients given the DNA damaging nature of psoralen (*e.g.* Lai *et al*, 2008; Honigsmann, 2001). In comparison our UVA split-dose strategy is likely to be much more effective with two low UVA doses especially that such UVA doses would be much less toxic with negligible cumulative effect.

As previously discussed, due to the proposed low doses of UVA applied in these experiments, the risk of UVA causing carcinogenicity is very low. It could be argued that the more effective ALA-PDT by UVA light could be more painful due to high generation of ROS, thus more ROS formation could generate more pain in treated patients. However to our knowledge there is no evidence that ROS formation in ALA-PDT is directly related to the pain experienced by the patients. For example blue light (within UVA range), is currently used in the US for treating AK and no study has directly related the ALA-PDT mediated pain to the choice of blue light. Nevertheless the relationship between ROS formation and the pain induced by the patients during ALA-PDT by UVA may be clinically investigated in the future.

The potential of fractionated illumination with red light in ALA-PDT has already been demonstrated in a range of preclinical studies in a variety of clinical models (*e.g.* de Bruijn *et al*, 1999; Robinson *et al*, 2000). These studies show that fractionated PDT results in significantly higher responses compared with PDT performed with a single illumination (*e.g.* de Bruijn *et al*, 1999; Robinson *et al*, 2000). Such studies have also shown the importance of the time interval between ALA application and the first light fraction and its relation to the synthesis of PPIX. For example Robinson *et al* (2003) have shown that a fractionated illumination scheme in which a cumulative fluence of 100 J/cm^2 red light is delivered in two equal light fractions separated by a dark interval of 2h is considerably more effective in ALA-PDT of SHK1 HR hairless mouse than one single dose. They have further demonstrated that the efficacy of such a scheme is further increased if the fluence of the first light fraction

is reduced to 5 J/cm^2 , since the photobleaching of PPIX during the first illumination and its re-synthesis in dark interval would contribute to the effectiveness of the second dose. Although in our study, we did find that a lower first dose was not effective in UVA-based split-dose ALA-PDT of HaCaT cells, we could not exclude the possibility that the effectiveness of our higher UVA split-dose combinations is due to re-synthesis of PPIX during the 1h dark interval. So it is necessary that in future studies the PPIX level as well as the kinetics of PPIX fluorescence be investigated under our experimental conditions. However they have also found that these regimes do not work with MAL, which is the licensed drug in the UK.

Besides the influence of the fluence rate of the individual light fractions, the time interval between light fractions has been shown to be critical in ALA-PDT with red light. For example enhanced ALA-PDT response is achieved if two lights fractions are delivered 4 and 6h after the application of ALA, separated by a 2h dark interval (de Bruijn et al, 2006). In a recent clinical study performed by de Haas *et al* (2008) involving 552 patients with sBCC, nBCC, BD and AK, a two fold illumination scheme was carried out using three different light sources (i.e. one diode laser at 630 nm and two broadband light sources with spectral output of 590-650 nm and 633 nm with a bandwidth of 20 nm, respectively). The protocol involved the topical application of 20% ALA for 4 h followed by two light fractions of 20 and 80 J/cm^2 separated by a 2 h interval. After an average follow up of 12-24 months, an overall complete response of 95% was seen for all lesions. So based on these findings, it is necessary that in future studies, comparative studies be carried out to evaluate the impact of the length of ALA treatment as well as the time interval between UVA split doses in improving the efficiency of UVA-split dose ALA-PDT in our experimental model. Such

studies are necessary to establish standard protocols that then could be evaluated in animal studies in order to find out the most efficient conditions for the use in clinic.

Finally, while our pilot study presented here provides experimental evidence of the high effectiveness of split-dose UVA strategy for ALA-PDT, in future studies it will be necessary to repeat these data with MAL, as ALA is not approved in Europe for PDT of skin superficial lesions such as AK since it has not being patented. The further tuning of the UVA-split protocols involving topical MAL or ALA in animal studies, could then be tested in pilot clinical studies involving patients with superficial AK lesions. Although long follow-up times will be required to assess potential procarcinogenic effects, these comparative studies may provide further insights into the effectiveness of either treatments for AK as well as the suitability of ALA- or MAL-based PDT in decreasing the pain experienced by the patients during the therapy.

CHAPTER FIVE

References

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6. Figure References

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